

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**Role of Sulfate Half-Ester Groups on the Oxidative
Modifications and Network Formation
of Cellulose Nanocrystals**

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Cover: Suspension of Green Cellulose Nanocrystals with Sulfate Half-Ester Groups Represented by Small Red Spheres on the Surface (Image created by Bing image creator using AI)

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ABSTRACT

Cellulose nanocrystals (CNCs) are nanomaterials with unique mechanical, physical, and chemical properties, which make them suitable as films and foams. To tap into the potential of CNCs and develop materials with tailored properties and a wider range of applications, it is essential to overcome existing challenges. These include surface-specific chemical modification, detailed analysis of functional groups, and stable CNC dispersion in water without aggregation. The present doctoral thesis addresses these challenges by investigating the role of sulfate half-ester groups, found on CNC surfaces, and in the chemical or enzymatic oxidative modification of CNCs. Moreover, the thesis explores the properties of CNC networks in water suspensions and as part of gel matrices. The results reveal that sulfate half-ester groups, which are negatively charged and bulky, have varied effects on oxidation reactions, highlighting the need to consider functional groups when working with nanomaterials. Furthermore, the thesis emphasizes the importance of analyzing both solid and soluble products for accurate conclusions. The work presented herein also illustrates the methods used to modify the CNC-CNC interaction governed by sulfate half-ester groups. The findings in this thesis advance our knowledge of CNC modifications, as well as our understanding of the mechanisms underlying CNC network formation. This will contribute to the development of green nanotechnologies rooted in nature's renewable resources.

Keywords: CNCs modification, periodate oxidation, enzymatic oxidation, rheological properties of CNCs, colloidal stability, trivalent cations, co-dispersant, CNC gelatine emulsion gels

LIST OF PUBLICATIONS

This thesis is based on the following appended papers:

I, The effect of sulfate half-ester groups on cellulose nanocrystal periodate oxidation

Saül Llàcer Navarro, Koyuru Nakayama, Alexander Idström, Lars Evenäs, Anna Ström and Tiina Nypelö

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II, Fat tissue equivalent phantoms for microwave applications by reinforcing gelatin with nanocellulose

Hana Dobšíček Trefná, Saül Llàcer Navarro, Fredrik Lorentzon, Tiina Nypelö and Anna Ström

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Saül Llàcer Navarro, Eliott Orzan, Ratchawit Janewithayapun, Paavo Penttilä, Anna Ström, Roland Kádár, and Tiina Nypelö

Manuscript

V, Sulfated cellulose nanocrystals hosting trivalent lanthanide cations Eu^{3+} , Gd^{3+} and Dy^{3+} offer new bioimaging tools

Panagiotis Spiliopoulos, Saül Llàcer Navarro, Eliott Orzan, Reza Ghanbari, Rudolf Pietschnig, Clemens Stilianu, Andreas Schaefer, Roland Kádár and Tiina Nypelö

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CONTRIBUTION REPORT

Paper I

Main author. I worked closely with co-authors to design the experiments.

I executed all of the experiments, with the exception of SAXS, NMR spectroscopy, and XPS. I took the lead in writing the first draft of the manuscript and carried the responsibility for the final version of the manuscript.

Paper II

Co-author. Co-performed the development of the phantom formulation and performed the rheological characterization.

Paper III

Shared first authorship. I was responsible for analysis of the solid fraction and data analysis. Wrote the first draft of the manuscript and was co-responsible for writing the manuscript.

Paper IV

Shared first authorship. I led the experimental design, planned the experiments, was responsible for the rheological characterization, participated in data analysis, wrote the first draft of the manuscript, and was responsible, together with my co-author, for writing the manuscript.

Paper V Co-author. Performed zeta-potential and thermal gravimetric analyses, participated in data analysis, reviewing, and editing.

Nomenclature

[COOH]	Concentration of carboxyl groups	mol COOH/g of CNC
ω	Angular frequency	rad/s
c_{NaOH}	Concentration of the NaOH solution	mol/L
G'	Storage modulus	Pa
G''	Loss modulus	Pa
k'	Reaction rate constant	1/s
m_{CNC}	Mass of CNCs	g
R_s	Surface ratio	
t	Time	s
V_{NaOH}	Volume of NaOH used for neutralization COOH	mol/L
[P]	Periodate concentration	mol/L
AFM	Atomic force microscopy	
AGU	Anhydroglucose unit	
CBM	Carbohydrate-binding module	
CNCs	Cellulose nanocrystals	
CNFs	Cellulose nanofibers	
DAC	Dialdehyde cellulose nanocrystals	
DeCNC	Desulfated CNCs	
DeDAC	Desulfated dialdehyde cellulose nanocrystals	
DO	Degree of oxidation	%

Nomenclature

DSS	Degree of surface substitution	%
DSS _{ox}	DSS of oxidated AGUs	%
DSS _s	DSS of AGUs with sulfate half-ester group	%
DSS _U	Degree of surface sites used	%
LPMO9	<i>Tt</i> LPMO9G	
NaOH	Sodium Hydroxyde	
NE	Enzymatic reaction without LPMO9	
NMR	Nuclear magnetic resonance	
NR	Enzymatic reaction without reductant	
T	Temperature	°C
tan δ	Tangent of the phase angle	
TBA	Tert-butanol	
UV	Ultraviolet	
XPS	X-ray photoelectron spectroscopy	

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To my family...

1

Introduction

Nanocelluloses represent a class of nanomaterials derived from cellulose biomass, and whose size is smaller than 100 nm in at least one dimension [1]. Nano-scale fibrils and rod-shaped particles result from the deconstruction of the hierarchical order typical of cellulose fibers [2]. Nanocelluloses exhibit some interesting properties, including high aspect ratio, high specific mechanical strength, semi-crystalline structure, high surface area, and the ability to form networks in aqueous media [3, 4].

Cellulose-based materials have emerged as a key solution in the field of material science, primarily due to their inherent sustainable and renewable nature [2]. This aspect is becoming increasingly important giving the impending environmental challenges and need for sustainable alternatives to traditional non-renewable materials. The drive for cellulose-based nanomaterials is in line with the United Nations Sustainable Development Goals 9, 12, and 13, which focus collectively on industry, innovation, infrastructure, responsible consumption and production, and climate action [5]. The versatility and adaptability of nanocelluloses has resulted in the filing of nearly 4,500 patents related to cellulose nanomaterials between 2010 and 2017 [1]. Consequently, nanocelluloses have assumed an important role in advancing the field of material science, opening up a vast array of opportunities, as well as innovative and sustainable solutions.

There are various types of nanocellulose materials, including cellulose nanocrystals (CNCs), bacterial nanocellulose, algal cellulose, and cellulose nanofibers (CNFs) [6]. CNCs are rod-like nanoparticles obtained after hydrolyzing cellulose biomass and retaining the crystalline cellulose regions [7]. Typically, CNCs range from 100 to 300 nm in length and from 5 to 10 nm in width [7]. Moreover, CNCs have high crystallinity when compared to other cellulose materials,

high specific Young's modulus¹ [8], and high specific surface area. However, the properties of CNCs depend on both their source and the selected manufacturing process. Industrially available or commercial CNCs are typically produced from cellulose using sulfuric acid (H_2SO_4), which hydrolyses² less ordered or amorphous regions of cellulose elementary fibrils and esterifies some of the hydroxyl³ groups present on superficial cellulose molecules⁴[9]. This process results in nanoparticles with a crystallinity index above 70%⁵ [11], and whose surface is composed primarily of hydroxyl and randomly distributed sulfate half-ester groups [12].

The presence of sulfate half-ester groups on the surface of CNCs influences their properties, including surface charge, thermal and colloidal stability, dispersivity in water, as well as possible modifications. Accordingly, sulfate half-ester groups on CNCs may decrease the hydrolysis rate of certain enzymes, regulate the nucleation of silver nanoparticles, and hinder fluorescence labeling for analytical purposes [11, 13, 14, 15]. Sahlin et al. [16], exploited the sulfate half-ester groups by having them react with azetidinium salts, thereby augmenting the hydrophobicity of CNCs and fine-tuning other material properties based on the type of azetidinium ion⁶ [17].

Cellulose nanocrystals have abundant hydroxyl groups and reducing ends⁷, both of which can be chemically modified [18, 19, 20]. Oxidation is an established modification approach. For example, modification via periodate oxidation introduces aldehydes⁸ on the CNC surface, resulting in dialdehyde cellulose [21]. Aldehydes have been used to crosslink networks or react with other functionalities, generating, for instance, UV-absorbing molecules [22]. Cellulose can be modified also via addition of carboxyl⁹ groups. Carboxyl groups are added to

¹Young's modulus is a measure of how stiff a material is.

²Hydrolysis is a chemical reaction that breaks chemical bonds.

³A hydroxyl group has a hydrogen atom covalently bonded to an oxygen atom.

⁴Resulting in the formation of the negatively charged sulfate half-ester groups (R-O-SO_3^-) on those hydroxyls.

⁵Determined by the Segal method [10].

⁶An ion is an atom or group of atoms that carries a positive or negative electric charge.

⁷Reducing ends are the ends of the cellulose chains that have a free aldehyde group.

⁸A aldehyde group has a carbon atom double-bonded to an oxygen atom (carbonyl group) and single-bonded to a hydrogen atom.

⁹A carboxyl group (COOH) has a carbon atom double-bonded to an oxygen atom (carbonyl group) and a single-bonded to a hydroxyl group.

nanocellulose via TEMPO-mediated oxidation [23], weak acid hydrolysis [24] or ozone oxidation [25]. While chemical reaction pathways enable efficient carboxylation procedures, their reliance on toxic and environmentally damaging reagents makes them unsuitable for medical applications or a green economy [26]. Enzymatic modifications present a viable alternative in this context. Even though enzymatic hydrolysis focuses predominantly on the degradation of cellulose biomass, as in the case of cellulases employed in the chemical and fuel industries, recent efforts have focused also on the use of enzymes to modify nanocelluloses [27, 28, 29].

One of the most interesting features of nanocelluloses is their ability to form networks in water via electrostatic, hydrogen, and covalent interactions [2, 30]. Electrostatic interaction is based on the attraction between opposite charges on nanocellulose and other molecules or ions [31]. Hydrogen interaction is based on the formation of hydrogen bonds between hydroxyl groups on nanocellulose and other polar molecules, such as water or alcohols. The third type is covalent interaction, which is based on the formation of covalent bonds between reactive groups on nanocellulose and other molecules [31]. These networks can be then modulated by increasing the ionic strength¹⁰ of the system, through addition of other solvents and surfactants¹¹, and by inducing covalent bonds between nanocellulose units and/or with other polymers [32, 33, 34, 35, 36, 37, 38]. Alterations in the surface charge of nanocelluloses can also affect network structure, as observed after increasing the number of carboxyl groups in carboxyl CNFs [39]. Chau et al. [32] showed that an increased cation charge led to stronger gels due to decreased electrostatic repulsions among CNC particles. This enabled the dominance of Van der Waals and hydrogen bonding¹² interactions, which changed the rheological properties. However, the presence of ions or surfactants as additives can have also a negative impact on the final properties of the resulting materials. Co-solvents that modify the suspension's properties, but which can also be removed during the generation of CNC solid foams, could

¹⁰Ionic strength indicates ion concentration in a solution.

¹¹Surfactants are molecules that have both a part that likes water (hydrophilic) and a part that dislikes water (hydrophobic). Surfactants can reduce the interfacial tension between two immiscible phases, such as water and oil.

¹²Hydrogen bonding in cellulose is a type of attraction between the hydroxyl groups, it occurs both within and between the cellulose chains/particles.

help overcome this issue. Kimura et al. [40], Winkler et al. [41], and da Silva et al. [42] have shown that alcohol¹³ co-solvents can modify nanomaterials networks by various mechanisms, such as solvent structuring, hydrophobicity, and the formation of networks or microemulsions. Such interventions resulted in anomalous viscosity behavior, nanoparticle assembly, and enhanced surface area.

This thesis advances our understanding of wood nanoscience and nanotechnology, by focusing on two main topics. The first topic centers around the role of sulfate half-ester groups in the modification of commercially available CNCs by periodate or enzymatic oxidation. It is based on the hypothesis that these negatively charged and bulky groups can hinder oxidation reactions and influence the resulting products. Accordingly, the modified products were characterized to evaluate their degradation, colloidal stability, and final composition. The second topic revolves around the different mechanisms involved in CNC network formation. Here, the hypothesis is that the addition of trivalent cations and a co-dispersant decreases the repulsion between CNCs caused by sulfate half-ester groups and enables the formation of networks. The findings in this thesis will help improve the modification and utilization of commercially available CNCs, as well as the formation of CNC networks suitable for sustainable and eco-friendly nanotechnologies.

¹³An alcohol is a compound that has a hydroxyl group attached to a carbon atom.

2

Background

2.1 CNCs and their surface properties

Cellulose is a linear biopolymer consisting of glucose, i.e. anhydroglucose units (AGUs), linked together by β -1,4-glycosidic bonds [43]. Each AGU in the cellulose chain has three hydroxyl groups located at carbon 2, 3, and 6 (C2, C3, and C6), which are available for hydrogen bonding and chemical modification (Fig. 2.1).

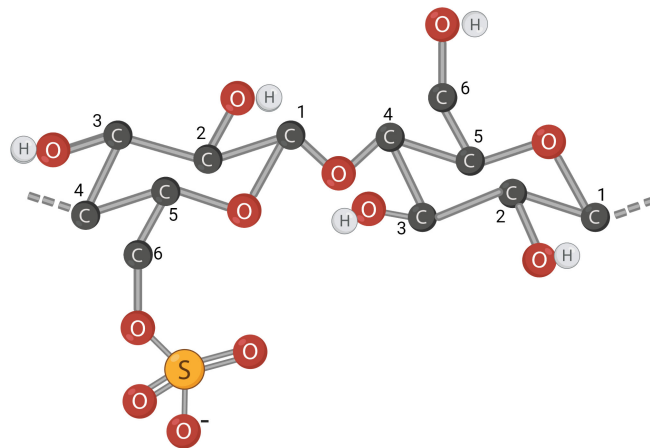


Figure 2.1: Illustration summarizing the structure of cellulose, in which two anhydroglucose units (AGUs) are linked by a β -1,4-glycosidic bond and the carbon atoms are numbered. The glycosidic bond connects AGUs at carbon 1 (C1) and carbon 4 (C4) of the adjacent AGU in the cellulose chain. A sulfate half-ester group is shown attached to carbon 6 (C6).

A key feature of cellulose fibers is their semi-crystalline nature, as they harbor both crystalline and amorphous regions. The semi-crystalline structure of cellulose has been a subject of debate, with various models describing its organization. Yue et al. [44] and Habibi et al. [7] refer to a model in which cellulose

consists of successive crystallites and amorphous regions. Wichkholm et al. [45] propose a semi-crystalline structure that results from crystalline core-amorphous shell pairing along the cellulose chains. Usov et al. [46] argue that the kinks observed in CNF, traditionally attributed to the transition between crystalline and amorphous regions, are more likely the result of mechanical processing during sample preparation. They further substantiate the core-shell model through measurements of Young's modulus and other studies [45, 47, 48]. To this end, a recent study by Willhammar et al. [49] using scanning electron diffraction to probe the packing of polysaccharide¹ chains across CNF revealed local ordering of the chains in twisting sections.

Crystalline regions from cellulose fibers can be isolated in the form of CNCs. The cellulose fibers used in the manufacture of CNCs have been obtained from wood pulp, cotton, and agricultural or food industry waste [50]. Conventional methods to produce CNCs employ acid hydrolysis and oxidation. Alternative approaches include ionic liquids, enzymatic hydrolysis or sub-critical water. Some of them allow the introduction of specific functionalities on the CNC surface [51]. CNCs obtained via sulfuric acid hydrolysis are characterized by the presence of sulfate half-ester groups on their surface [52]. A sulfate half-ester group has a sulfur atom double-bonded to two oxygen atoms and single-bonded to another two oxygen atoms, (Fig. 2.1). In the case of cellulose, one of the single-bonded oxygens of the sulfate half-ester group is covalently bonded to a carbon, replacing one of its hydroxyl groups on C2, C3 or C6. However, the distribution of sulfate half-ester groups among these carbons remains unclear. Given the size and charge of the sulfate ion² and the smaller carbon-carbon distance in the AGU³, steric hindrance is likely to limit the reaction with hydroxyl groups at carbons C2 and C3. Moreover, the hydroxyl group at C6 is known to be the most reactive [55], while the ones at C2 and C3 are less reactive. Considering all these factors, it is reasonable to assume, for the purpose of this thesis, that the sulfate half-ester groups reside predominantly on C6. CNCs generated through sulfuric acid hydrolysis from wood pulp typically contain 240-330 mmol sulfate half-

¹A polysaccharide is long chain of many sugar units joined by chemical bonds (such as starch or cellulose).

²Size of the sulfate ion is 0.242 ± 0.007 nm [53].

³Carbon-carbon average distance is 0.15 nm [54].

esters per kg of CNC [56]. In addition, CNCs extracted by HCl or sulfuric acid hydrolysis contain small amounts of carboxylate groups⁴ [57, 58]. The amount of sulfate half-ester groups can be increased by adding sulfuric acid and heating the system for 2 h at 40 °C, or decreased by adding HCl at 80 °C with constant stirring for 2.5 h [57]. Sulfate half-ester groups provide a net negative charge at pH > 2 [59], which is important for dispersion in water, colloidal stability, and possible downstream applications [60]. Notably, however, the presence of sulfate half-ester groups decreases the thermal stability of CNCs [61].

CNCs harbor H⁺ counterions on their sulfate half-ester groups (protonated CNCs), but these counterions are commonly exchanged with Na⁺ ions (CNCs in Na⁺ form) to improve solubility in water [62, 9]. By controlling the drying process and managing the counterion exchange, both protonated CNCs and CNCs in Na⁺ form can be effectively dispersed in water [62].

In contrast, hydroxyls and the sulfate half-ester groups of the CNCs' surface lead to incompatibilities and low dispersibility of CNCs in hydrophobic media and matrices [63]. The main obstacle to keeping CNCs well dispersed in simple organic solvents such as alcohols is the dependence of CNC stability on electrostatic forces. These forces are weak in non-polar organic solvents and additional components are needed to prevent the nanocrystals from aggregation [64].

2.2 Chemically and enzymatically catalyzed modification of cellulose

The abundant surface hydroxyl groups located at C2, C3, and C6 make the CNC hydrophilic, and prone to hydrogen bonding and aggregation [65]. While these hydroxyl groups favor some interactions, their inherent properties may not be sufficient or ideal for all targeted applications. Therefore, functionalizing CNCs by modifying the surface hydroxyl groups can tailor the material's properties to suit specific applications [66, 67, 20]. Periodate oxidation cleaves the C2 - C3 bond in the AGU, transforming both carbons into aldehydes (Fig. 2.2). These aldehydes can be further hydrated in water to form hemiacetals⁵ or hemialdals⁶,

⁴20–30 mmol carboxylate groups per kg of CNC.

⁵A hemiacetal is a compound that results from the addition of an alcohol to an aldehyde.

⁶A hemialdal implies the presence of a C3–O–C2 bond.

2. Background

and then further reduced to alcohols or carboxyl derivatives [68, 69, 70, 71]. These derivatives can be used to alter the thermal and mechanical properties of cellulose materials [72, 73]. However, periodate-induced chemical modification is often accompanied by degradation [66]. The crystallinity of cellulose decreases with periodate oxidation, and temperature accelerates both oxidation and degradation [74]. Periodate oxidation of CNCs proceeds heterogeneously, leading to local loss of order and isolated oxidized domains or clusters. Highly ordered regions in cellulose become affected even at a low degree of oxidation (DO) [75, 76]. A result of periodate oxidation is peeling off of the surface layer, as evidenced by altered CNC dimensions [77] and nuclear magnetic resonance (NMR) spectroscopy [78]. Overall crystallinity is a determining factor of the periodate oxidation rate [79].

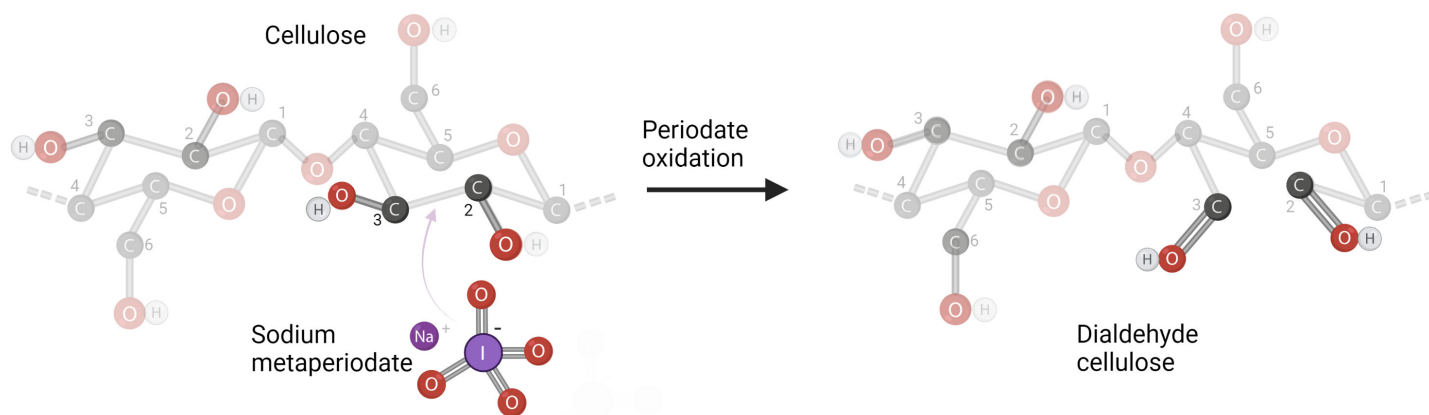


Figure 2.2: Illustration of the periodate reaction on cellulose. The left side shows a cellulose molecule attacked by metaperiodate. On the right side, after periodate oxidation, the bond between carbon 2 and 3 is broken producing dialdehyde cellulose. Dialdehyde cellulose has an aldehyde group residing at each carbon 2 and 3.

The manufacturing of CNCs through sulfuric acid hydrolysis involves harsh reaction conditions, large amounts of water, and numerous purification steps, all of which have a negative environmental impact [52, 80]. To address these issues, alternative eco-friendly methods for producing CNCs have been attempted. One promising approach is based on the use of enzymes that selectively digest the less ordered regions of cellulose and release CNCs [81]. Besides diminishing the environmental impact, this method provides also a targeted way of modifying

CNCs for various downstream applications [27].

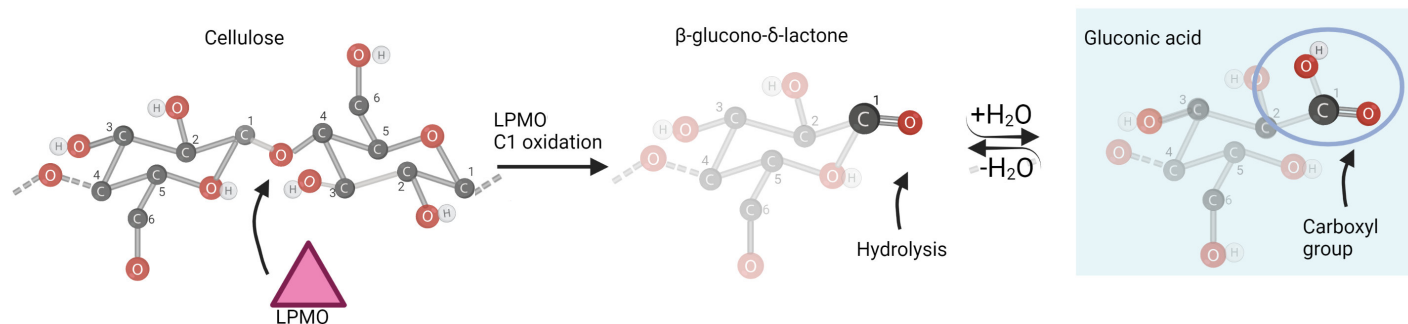


Figure 2.3: Illustration of the LPMO C1-oxidation on cellulose. On the left, the glycosidic bond is targeted by an LPMO (depicted as a triangle). Following LPMO C1-oxidation, a hydrolysis event at the glycosidic bond leads to the production of a β -glucono- δ -lactone molecule and a separate cellulose molecule. The β -glucono- δ -lactone exists in equilibrium with gluconic acid in water. On the right, the carboxyl group on gluconic acid obtained through LPMO oxidation is highlighted.

Lyctic polysaccharide monooxygenases (LPMOs) are relevant from an industrial lignocellulosic biorefinery perspective as they offer mild and sustainable enzyme-based alternatives to chemical and mechanical reaction pathways [82, 83]. LPMOs are regiospecific mono-copper carbohydrate-active enzymes that cleave polysaccharides at various positions (C1, C4, and C6) [84, 85, 86, 87]. LPMOs efficiently catalyze carboxylation at C1 by creating β -glucono- δ -lactones⁷ in equilibrium with gluconic acids⁸ (Fig. 2.3), or at C4 by creating 4-keto sugars⁹ in equilibrium with gemdiols¹⁰. Catalysis by LPMOs requires Cu(I) in the active site, which is obtained through reduction of Cu(II) by an electron donor or reductant such as ascorbic acid, as well as an oxygen-containing co-substrate, such as O₂ or H₂O₂ [82, 88, 89]. LPMOs have a flat active site and can create nicks on crystalline and recalcitrant polysaccharides, allowing for increased saccharification yields of various substrates [90].

Cellulose carbohydrate-binding modules (CBMs) are non-catalytic domains that promote enzyme binding to a substrate and are connected to the catalytic

⁷An AGU that C1 has double-bonded oxygen or a carbonyl group.

⁸An AGU that C1 has a carboxyl group.

⁹An AGU that C4 has a carbonyl group.

¹⁰An AGU that C4 has two hydroxyl groups.

domains via a linker. The CBM promotes localized and repeated oxidation of the substrate by binding to specific regions of the polysaccharide chain. A repeated attack in localized areas of a chain could be related to the oxidative cleavage of cellulose chains by LPMOs [91, 92, 86].

LPMOs vary with respect to substrate preference, modularity, regioselectivity, and reaction kinetics. Family AA9 is a family of LPMOs with proven activity on cellulose, various hemicelluloses, and the respective oligomers [93]. LPMOs from family AA9 contain a CBM [94], and are industrially interesting because they can boost cellulolytic activities in commercial lignocellulolytic cocktails. Family AA9 has also been used to produce and oxidize cellulose nanomaterials in combination with cellulases and xylanase [95, 96, 97, 98, 99, 100, 101, 102, 103, 104]. *TtLPMO9G* or *LPMO9* is a C1-oxidizing AA9-family LPMO from *Thermothielavioides terrestris* [105]. The enzyme consists of a C1-oxidizing catalytic domain fused to a CBM domain. Previous studies have demonstrated the strong and highly specific activity of *LPMO9G* on various cellulosic substrates [105].

2.3 Challenges of analyzing functional groups

The type and number of functional groups are important analytical factors when modifying CNCs. While elemental analysis is a reliable method for determining the amount of sulfur in CNCs, it does not provide information on their location. Assuming that each sulfur atom is associated with a sulfate half-ester group on the surface of the CNC, sulfur content can be directly translated into sulfate half-ester content [56]. Fourier transformed infrared spectroscopy is commonly used to identify functional groups, but it has its limitations due to the presence of water and impurities that can obscure the spectra, and it cannot offer any cues on the location of functional groups.

X-ray photoelectron spectroscopy (XPS) is a surface-sensitive technique that can provide information on the elemental composition of the CNC surface, including the presence of functional groups [106]. However, it requires a vacuum environment and may be affected by charge build up on the cellulose surface [107, 106]. Furthermore, the presence of other functional groups or contaminants on the CNC surface, may interfere with the O 1s and C 1s peaks obtained

by XPS [108].

Conductometric titration with NaOH is commonly used for the determination of CNC surface charge and the degree of substitution of carboxyl groups. As this method measures protons associated with sulfate half-esters rather than the charged groups themselves [56], when used for the determination of carboxyl groups, it detects the presence of a weak acid rather than the carboxyl group only. Moreover, differentiating between sulfate and carboxyl groups can be challenging when the concentration of carboxyl groups increases, as this can affect the dissociation constants and obscure the determination of the first equivalence point [109]. Thus, titrating CNCs with NaOH can provide an estimate of the degree of substitution, but it is not as accurate as other methods such as NMR spectroscopy.

NMR spectroscopy provides information about the chemical environment of individual atoms in a molecule and can be used to distinguish between different types of functional groups [78]. NMR spectroscopy is a non-destructive technique and does not require any chemical modification of the sample. However, NMR spectroscopy can be time-consuming, and expensive. In addition, the interpretation of the data can be complicated by the presence of impurities and overlapping signals.

To overcome the limitations of existing analytical methods, it is often wise to use a combination of techniques. Incorporation of different analytical techniques allows us to obtain a more complete picture of the modifications and ensures a comprehensive analysis of the functional groups.

2.4 Hierarchical structuring of cellulose suspensions

Colloidal stability represents the ability of particles to remain homogeneously dispersed in a suspension without aggregation or sedimentation. Colloidal stability is crucial for CNCs, as their aggregation may hinder processing and cause the loss of properties such as birefringence [110]. Therefore, understanding which factors influence the colloidal and rheological properties of CNCs is of paramount importance.

The colloidal stability of CNCs is governed by interparticle interactions, which are influenced by pH, ionic strength, surface charge, and surface functional

groups [111]. Colloidal stability can be estimated from the value of the zeta potential. The latter represents the electrical potential at the slipping plane, which is the interface separating the mobile fluid from the fluid that remains attached to the surface [111, 112]. That potential is related to the dissociation of surface groups or adsorption of ions from the medium, which reflects the electrostatic repulsion between particles [111, 112].

The zeta potential provides a qualitative assessment of charged functional groups. Stability of colloidal systems can be categorized by the magnitude of zeta potential: systems with zeta potential values of ± 20 -30 mV are moderately stable, while those exceeding ± 30 mV tend to exhibit high stability [111]. Conversely, systems with zeta potential values in the range of ± 0 -20 mV are typically unstable and prone to particle aggregation and sedimentation. It is essential to remember that these stability thresholds are not constant but depend on factors such as temperature. In fact, the electrostatic potential should overcome the thermal energy to ensure colloidal stability. Notably, the zeta potential does not correspond to the charge content of any specific functionality on the surface of CNCs, but reflects instead the overall surface charge.

Colloidal stability has a significant impact on the rheological properties of suspensions. In CNC suspensions, the surface charge of the particles can influence their ability to repel or attract each other. On the one hand, when particles are sufficiently repulsive (zeta potential exceeding ± 30 mV), they tend to remain well-dispersed, which can lower the viscosity and favor a more fluid-like behavior. On the other hand, at lower repulsion (zeta potential ± 20 -30 mV), the particles can interact, leading to a higher viscosity and network formation [113]. A third scenario can arise when CNCs aggregate and precipitate (zeta potential ± 0 -20 mV) decreasing the viscosity [113].

The rheological properties of suspensions are influenced not only by the colloidal stability of CNC particles, but also by their size, shape, and concentration [114]. Viscosity represents a material's resistance to flow under an applied force and is typically measured using rotational deformation. The storage modulus (G') measures the elastic or recoverable energy stored in a material subjected to oscillatory deformation, thus reflecting how much a material behaves like a solid or an elastic material when deformed. In contrast, the loss modulus (G''), measures the viscous or dissipating energy lost in a material when subjected to

oscillatory deformation, reflecting how much a material behaves like a fluid. The tangent of the phase angle δ ($\tan \delta$) represents the G'' to G' ratio. Values of $\tan \delta$ close to zero indicate a more elastic (viscoelastic solid) behavior, whereas values higher than one suggest a more viscous (viscoelastic fluid) behavior.

Factors such as temperature, pressure, and time can affect the shear viscosity measurement, which can be carried out under steady or transient conditions [114, 115]. The amplitude sweep measurement evaluates the G' and G'' as a function of strain amplitude at a constant frequency. Performed in the oscillatory mode, this measurement provides information about linear and nonlinear regions, yield stress, and strain hardening or softening. Additionally, the amplitude sweep measurement can uncover the onset of irreversible deformation, structural breakdown, or flow instability in a material [114, 115]. The frequency sweep measurement examines the G' and G'' of a material as a function of frequency at a constant strain amplitude. It is also performed in oscillatory mode and provides an insight into the molecular structure, relaxation time, and viscoelastic behavior of a material.

The rheological behavior of CNCs in suspension is modulated by their concentration. At concentrations below the percolation threshold, which typically ranges from 0.7 to 3 wt% depending on the CNC aspect ratio, CNC particles rotate freely, with minimal interactions between them [116, 117, 118]. In this regime, the suspension's viscosity remains constant at low shear rates, displaying Newtonian behavior typical of a viscoelastic liquid [119]. At percolation concentration, CNCs form a continuous network throughout the suspension, leading to substantial changes in rheological properties [116, 117, 118]. However, as the concentration of CNCs increases to approximately 3-5 wt%, the viscosity function may show a three-region profile, in which the viscosity decreases with increasing shear rate in each region [119]. This means that CNCs start to interact with each other and form liquid crystalline phases of the isotropic, nematic, and cholesteric type [120, 121, 7, 119]. These suspensions start to demonstrate a gel-like behavior. From approximately 5-7 wt%, the viscosity function becomes restricted to a single shear-thinning region. The suspensions at this concentration exhibit the characteristics of a viscoelastic solid, with a constant G' regardless of frequency and G' significantly higher than G'' [119]. Such ranges and structures arise from a combination of several factors, such as surface charge,

electrostatic repulsion, concentration, CNC aspect ratio, solvent-solute interactions, and the use of sonication [119, 121, 7]. Therefore, directing the hierarchical structuring of the CNC network is crucial for controlling the suspensions properties.

In this context, one of the strategies that have been employed to manipulate the structure of nanoparticle networks is to introduce alcohols as co-dispersants [42, 40, 41]. Tert-butanol (TBA) is a moderately amphiphilic¹¹ alcohol that can disrupt the hydrogen-bond network between water molecules, leading to the formation of TBA-water microstructures [122, 123, 124, 40]. When TBA is added to water, a bicontinuous microemulsion is formed at TBA concentrations between 0.3 and 0.85 molar fractions (18-64 wt% of TBA). Below this range, the hydrophobic effect would cause TBA to quickly devolve into TBA-rich domains dispersed in the aqueous phase [123, 122].

2.5 CNC-reinforced gelatine emulsion gels

Gelatine is a biopolymer derived from collagen that can form a self-standing gel when cooled below a certain temperature. Gelatine forms a three-dimensional network that traps water molecules by forming rigid triple helices connected by flexible links [125, 126]. The concentration of gelatine defines the mechanical properties of the gel, such as its shear modulus and strength [125, 126]. The resulting gel can be melted by heating above a certain temperature, which disrupts the triple helices and releases water [125, 126]. This melting process is reversible, although the melting and gelling temperatures are not the same. The typical range of melting temperatures for gelatine gels is 27–34 °C, irrespective of gelatine concentration [127]. The gelatine network can be reinforced by physical or chemical crosslinking to increase the melting point. Physical crosslinking relies on non-covalent interactions, such as hydrogen bonding and network entanglements; whereas chemical crosslinking involves the formation of covalent bonds between polymer chains [126]. An example of chemical crosslinking is the Schiff reaction, whereby the amine groups of gelatine react with the aldehyde groups. Aldehyde groups are present in dialdehyde cellulose, which has been

¹¹Amphiphilic is a term that describes a molecule that has both hydrophilic and hydrophobic parts.

proposed as a crosslinker for gelatine [128, 129]. Addition of dialdehyde cellulose replaces the need for more common but toxic crosslinkers such as formaldehyde¹². As an amphiphilic biopolymer [131], gelatine is an excellent candidate for reinforcing emulsions. Emulsions are dispersions of two immiscible liquids, whereby one is dispersed in a continuous liquid phase, such as oil and water [132]. In oil-in-water emulsions, oil droplets are dispersed within a continuous water phase [132]. A surfactant, which decreases the interfacial tension between the two immiscible liquids, is used to prevent droplets from coalescing [132]. Gelatine emulsion gels are rather stable, as the oil phase is evenly distributed within the water-gelatine matrix and strengthens the gel [133]. However, the ability to increase the melting point of gelatine emulsion gels without the use of toxic crosslinkers remains a challenge [134, 135].

¹²Acute oral toxicity (LC₅₀): 42 mg/kg (Mouse) [130].

3

Methodology

3.1 Modification of CNCs

Three grades of CNCs were used in this thesis work: commercial CNCs, protonated CNCs, and desulfated CNCs. Commercial CNCs, which were in sodium form and sulfated, were simply dispersed in deionized water without any further modification. In this thesis, these are referred to as CNCs. To obtain protonated CNCs, commercial CNCs were first dispersed in water and protonated by adding HCl until reaching pH 2, which allowed them to remain below the pK_a of sulfate half-ester groups [136]. The dispersions were then dialyzed using a spectrapore membrane until the pH of the dialysate reached the pH of the deionized water employed for dialysis. For the desulfation process, a 5 wt% CNC dispersion was mixed with 12 M HCl to obtain an acid concentration of 1.8 M. The suspension was then heated to 90 °C and stirred with reflux for 2 h. The suspension was dialyzed against deionized water to yield desulfated CNCs (DeCNCs).

Periodate oxidation was carried out as described by Dash et al. [128] with some modifications. A weight ratio of 0.7 sodium metaperiodate (NaIO_4) to CNCs or DeCNCs in 0.1 M acetate buffer at pH 4 and 50 mM ionic strength was used. The initial concentration of CNC grades was 3 wt%. The reaction was initiated by adding the periodate solution to the CNC suspension and was allowed to proceed for varying times. Glycerol was used to quench the reaction, and the dispersions were dialyzed to remove any residual periodate and glycerol. After the periodate oxidation, the dispersions containing dialdehyde cellulose nanocrystals (DAC) or desulfated dialdehyde cellulose nanocrystals (DeDAC) were stored at 4 °C until further use.

Protonated CNCs, referenced in this study as CNCs, and DeCNCs were modified by LPMO9. LPMO treatment was performed using a suspension of 2.6 wt%

CNC or DeCNC in a 50 mM BisTris-HCl buffer at pH 6.5 and room temperature with constant stirring. The enzyme concentration was 0.9 μ M and the concentration of the reductant, ascorbic acid, was 1 mM. Before the reactions, LPMO9 was incubated with a 0.5 equimolar amount of CuSO_4 on ice for a minimum of 30 min. Ascorbic acid was added last to initiate the reaction. The cellulose dispersions were reacted for 6 h and aliquots were taken at 2, 4, and 6 h. CNCs and DeCNCs after 6 h of LPMO reaction are referred to as CNC 6h and DeCNC 6h respectively. Control reactions were performed without the reductant (NR) or LPMO9 (NE) in otherwise equivalent conditions for 6 h. To deactivate the enzyme, the suspension was heated to 100 °C for 10 min, and the pH was lowered to 2. The enzymatically modified cellulose dispersions were then purified by dialysis against deionized water.

3.2 Determining the degree of CNC modification

To determine the elemental composition (sulfur and metal cations), freeze-dried suspensions were sent to Mikrolab Kolbe (Oberhausen, Germany) and analyzed in duplicate. The extent of periodate oxidation is represented by the degree of oxidation (DO) and corresponds to the number of modified AGUs per total number of AGUs. The DO of the periodate oxidation was tracked in-situ by monitoring UV absorbance at 290 nm, which indicated the consumption of periodate during oxidation [137]. Other methods, including NRM spectroscopy or hydroxylamine hydrochloride assay, were also used in Paper I to determine the DO of the periodate oxidation.

Conductometric titration was performed to quantify the carboxyl group content of CNCs. Before titration, CNC suspensions were protonated and dialyzed. The suspensions were diluted with ultrapure water, and HCl and NaCl were added to increase conductivity, as well as enable the identification of equivalence points. Titration was conducted using 0.01 M NaOH and a 665 Dosimat titrator controlled by a Python script. The equivalence points were identified using Matlab. The first equivalence point indicated neutralization of strong acid groups ($-\text{OSO}_3\text{H}$) and HCl excess; whereas the second indicated neutralization of weak acid groups ($-\text{COOH}$). At each titration point, the conductivity was calculated based on the measured value, the initial suspension volume, the volume

of NaOH added, and the dilution of the suspension. The concentration of NaOH was standardized using potassium hydrogen phthalate. The volume of the NaOH used for the neutralization of the weak acid groups (V_{NaOH}), combined with its known concentration (c_{NaOH}) and the CNC dry content (m_{CNC}), was then utilized to calculate the concentration of the carboxyl groups ($[\text{COOH}]$) (Equation 3.1).

$$[\text{COOH}] = \frac{V_{\text{NaOH}} \times C_{\text{NaOH}}}{m_{\text{CNC}}} \quad (3.1)$$

3.3 Degree of surface substitution

The surface ratio (R_s) was calculated as the ratio between the number of surface AGUs and the total number of AGUs in CNCs [138, 139] (Paper I and Paper III). The degree of surface substitution (DSS) quantifies the number of modified AGUs per surface AGU. DSS_{ox} refers to AGUs oxidized to either two aldehydes or carboxyl, while DSS_s denotes the sulfate half-ester functionality. Given the alternating availability of C2 and C3 in the cellulose polymer chain, it is assumed that only every other AGU on the surface can be modified by periodate oxidation. Consequently, the degree of surface sites used (DSS_U) is defined as the proportion of surface AGUs that were effectively oxidizable and subsequently modified. DSS_{ox} , DSS_s , and DSS_U were calculated using Equations 3.2, 3.3, and 3.4 respectively. In these equations, n_{ox} , n_{tot} , and n_{sur} represent the number of oxidized AGUs, the total number of AGUs, and the number of AGUs on the surface, respectively; while x_s represents the molar ratio of sulfur to AGU.

$$\text{DSS}_{\text{ox}} = \frac{n_{\text{ox}}}{n_{\text{sur}}} = \frac{\text{DO}}{R_s} \quad (3.2)$$

$$\text{DSS}_s = \frac{x_s}{R_s} \quad (3.3)$$

$$\text{DSS}_U = \frac{\text{DO}}{\frac{R_s}{2}} \quad (3.4)$$

3.4 Kinetics of the periodate oxidation reaction

The reaction of periodate with C2 and C3 on cellulose produces dialdehyde cellulose and occurs as a solid-liquid heterogeneous reaction on the surface of CNCs. To simplify the analysis, the following assumptions were made: the reaction followed pseudo-first-order kinetics, it was slow and was not limited by mass transfer or side reactions, and there was a linear dependence with the concentration of periodate. The reaction rate constant (k') was determined from the concentration of periodate ($[P]$) at time t relative to its initial concentration ($[P_0]$) using Equation 3.5.

$$\frac{[P]}{[P_0]} = e^{-k' \cdot t} \quad (3.5)$$

3.5 Analysis of CNC diameters

CNC diameters were measured by atomic force microscopy (AFM) in semi-contact mode by an atomic force microscope (NTEGRA, NT-MDT, Russia) equipped with Tap300AI-G tips, which recorded regions of interest ($5 \times 5 \mu\text{m}$ in size) of CNCs on a silicon wafer (Siegert wafers, Germany). The recorded images were post-processed using Gwyddion 2.56 to remove the polynomial background and row-align the images. Potential single CNC profiles were extracted from the processed image, with a cross-section consisting of 1,000 points. At least 50 profiles were selected to determine the diameter distribution of each CNC grade.

The height of the CNCs was calculated using Matlab 2019. The profiles were fitted with an arbitrary order to reduce noise; in this case, a polynomial of the 14th degree was chosen. The polynomial was differentiated, and the inflection points were listed, with only those with a positive change of sign being considered. The profile was divided into sections based on the inflection points, with the average height of each section calculated. The section with the highest average height was considered as the main section. Moving outwards from the main section, the average height of each adjacent section was compared to its inflection points. If the average height of the section was lower, the main section was increased until it reached the inflection point at the end of that section. The

inflection points of the main section were considered as the bottom points of the peak and were used for baseline correction, with the height being calculated perpendicular to the corrected profile. This methodology allowed for a reliable determination of the CNC diameter distribution.

3.6 Determination of colloidal stability

The zeta potential of dispersions containing CNC grades was measured using DTS 1070 cuvettes and a Zetasizer nano instrument (Malvern, UK). The measurements were carried out at 25.0 ± 0.1 °C, with CNC grades at a concentration of 0.25 wt% [140]. The data were processed using Zetasizer V7.13 software (Malvern). A total of six measurements were performed for each CNC grade, with each measurement consisting of 30 to 150 sub-runs. The average of the six measurements corresponded to the zeta potential.

3.7 Preparation of CNC networks

CNC dispersions with trivalent cations were prepared by dispersing 2.25 wt% commercial CNCs in ultrapure water. Lanthanide chloride solutions (1 wt%) in ultrapure water were added to the CNC dispersions to achieve a sulfur-to-lanthanide atomic ratio of 1:1. The dispersions were stirred overnight to ensure complete binding of the lanthanide ions to the sulfate half-ester groups on the CNC surface. The dispersions were then dialyzed through SpectraPore membranes with a cut-off of 12,000-14,000 kDa to remove any excess lanthanide ions and other impurities.

The CNC-water suspensions containing TBA as co-dispersant were prepared from a 10 wt% CNC stock suspension in MilliQ-water that was prepared using an IKA EUROESTAR 60 overhead stirrer with an R 1342 propeller stirrer (4-bladed) at 1200 rpm for 1 h. The suspensions were prepared following a design of experiments (Paper IV), and the stock suspension was diluted in water to the desired concentration. TBA was then added in seven increments, with the suspension being shaken after each addition to avoid heterogeneous or phase-separated suspensions. The suspensions were shaken overnight on a moving

platform at 180 rpm.

To prepare the gelatine emulsion gel reinforced with CNC and DAC, oil and water were separately heated to 60 °C. In the water phase, Lecithin B-10¹ (Dupont, Denmark) was dispersed and left to mix for 10 min. Bloom 296 Gelatine A (Gelita, Sweden) was then added and mixing was continued for an additional 20 min. The oil phase was added dropwise under vigorous stirring using an Ultra-turrax (IKA T50), resulting in a visually turbid system.

For the preparation of CNC/DAC (DO 14%) reinforced gelatine emulsion gels, the procedure was similar to that of gelatine emulsion gels, except that CNC or DAC dispersions were heated to 50 °C before being added to the highly concentrated gelatine-water system, followed by the dropwise addition of oil. The emulsions consisted of 70 wt% oil, 2 wt% gelatine, 0.6 wt% lecithin, 0.6 wt% CNC grade (if required), and water.

3.8 Rheological measurements

To assess the effect of trivalent cations on CNCs, a steady shear test was conducted using an Anton Paar MCR 702 Twin Drive rheometer (Graz, Austria) equipped with a P-PTD200/glass measuring cell in a single motor-transducer configuration. The parallel-plate/glass measuring geometry had a diameter of 43 mm, with a measuring gap of 1 mm. For shear viscosity measurements, the shear rate ranged from 0.01 to 1000 1/s. In addition, oscillatory shear tests were conducted with the same configuration, but with a 50 mm diameter plate geometry and a measuring gap of 1 mm. Strain amplitude sweep tests were conducted within a range of 0.01% to 1000% at a constant angular frequency of 6.28 rad/s. Linear viscoelastic frequency sweep tests were performed in a separate motor transducer configuration using two parallel-plate 50 mm measuring geometry operated in counter-oscillation mode. The angular frequency ranged from 0.1 to 70 rad/s at a constant strain amplitude of 0.25% based on strain sweep tests.

The rheological properties of CNC-water suspensions containing TBA as a co-dispersant were determined using a TA Discovery HR-3 rheometer (TA Instru-

¹Lecithin was used as a surfactant.

ments, USA). A sandblasted plate with a diameter of 40 mm and a measuring gap of 1 mm were used for suspensions containing CNCs. When the systems consisted of only water and TBA, a 40 mm cone with a truncation angle of 1° and a pre-set measuring gap of $26\ \mu\text{m}$ was used. After placing the solutions or suspensions in the measurement position, they were allowed to equilibrate for 300 s before performing the measurements. A Peltier lower plate maintained a constant measuring temperature of $20\ ^\circ\text{C}$. Shear viscosity measurements were performed at shear rates between 1 and 1000 $1/\text{s}$ (solutions without CNCs), and between 0.01 and 1000 $1/\text{s}$ (suspensions with CNCs). Amplitude sweep measurements were conducted at 6.28 rad/s, within a strain amplitude range of 0.01% to 1000%. Frequency sweep measurements were conducted within a frequency range of 0.1 to 100 rad/s, with a strain amplitude of 0.3%

Finally, the rheological properties of gelatine emulsion gels reinforced with CNC and DAC, were determined using a TA Discovery HR-3 rheometer. The geometry was plate-plate (40 mm in diameter) with a gap of 1 mm. The upper plate was equipped with a solvent trap and used with a custom-made evaporation chamber (TA Instruments). The samples were added to the plate at $60\ ^\circ\text{C}$, and the upper plate was lowered to the measurement position. The temperature was then gradually decreased to $10\ ^\circ\text{C}$ at a rate of $2\ ^\circ\text{C}/\text{min}$ and held for 300 s. The temperature was subsequently increased to $60\ ^\circ\text{C}$ at a rate of $2\ ^\circ\text{C}/\text{min}$. G' and G'' were recorded at 6.28 rad/s and a strain of 0.5%. The sol-gel transition was determined by the point where G'' crossed G' [141]. The temperature was regulated using a Peltier plate.

4

Results and Discussion

The results and discussion section presents the experimental work carried out to investigate the effect of sulfate half-ester groups on CNCs. Specifically, the experiments assessed the reactivity and ability of CNCs to be subjected to oxidative modifications (by periodate and LPMO9), as well as the potential of CNCs for use in network formation and thermal stabilization of gelatine. The results are divided in five parts: 4.1 compositional analysis of CNCs subjected or not to further modifications; 4.2 impact of sulfate half-ester groups on reaction kinetics during periodate or LPMO9 oxidation; 4.2.3-4.2.5 characterization of the oxidized products in terms of degradation, colloidal stability, final composition, and degree of substitution; 4.3.1 impact of trivalent cations and TBA as co-dispersant on the formation of CNC networks as well as the rheological properties of CNC suspensions; and 4.3.2 the potential of CNCs and DAC to increase the thermal stability of gelatine emulsion gels.

4.1 Compositional analysis of CNCs

Surface composition plays a crucial role in determining the properties and behavior of CNCs. In particular, the presence of sulfate half-ester groups on the surface of CNCs is thought to impact their reactivity and functionalization. Understanding how sulfate half-ester groups are distributed, both qualitatively and quantitatively, is essential for interpreting their role.

In this thesis, CNCs containing two different sulfur concentrations were used and their composition was determined by elemental analysis. The resulting information helped investigate the role of sulfate half-ester groups in periodate or LPMO9 oxidation. One grade was represented by both commercial CNCs and protonated CNCs, and the other one was its desulfated version obtained via acid

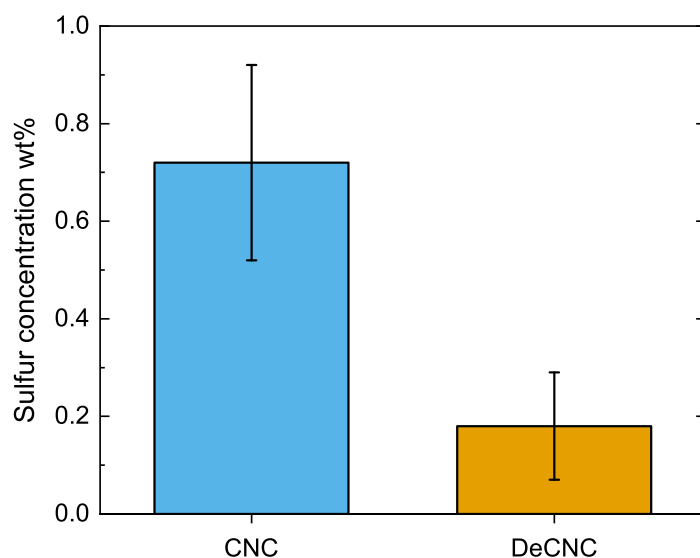


Figure 4.1: Sulfur concentration on CNCs and DeCNCs. DeCNCs were obtained via acid catalysis.

catalysis, which removed some of the sulfate half-ester groups [142]. Commercial CNCs in Na^+ form were employed for periodate oxidation as the use of sodium metaperiodate as the oxidizing agent made deprotonation unnecessary. For the enzymatic study, the protonated forms of CNCs and DeCNCs¹ were used to avoid any potential effects of sodium ions on LPMO9 activity.

Even though the sulfur content of CNCs may vary from batch to batch, it clearly decreased following the desulfation process. As shown in Fig. 4.1, the average sulfur content of CNCs was 0.72 ± 0.20 wt%, while that of DeCNCs was 0.18 ± 0.11 wt%. Therefore, it is essential to determine the sulfate content of CNCs each time they are used, particularly when studying sulfate half-ester groups. This practice can aid in the interpretation of results and provide an estimate of the deviation within the material and experimental data.

¹Desulfation also leads to protonation.

4.2 Effect of the sulfate-half-ester group on oxidative modifications of CNCs

The impact of sulfate half-ester groups on the reactivity and functionality of CNCs during oxidative modification via periodate or enzymes is discussed in this section. These modifications involve the introduction of carbonyl and carboxyl groups on the CNC surface respectively.

4.2.1 Effect of the sulfate-half-ester group on periodate oxidation of CNCs

The kinetics of periodate oxidation of CNCs was recorded by following the UV absorbance of the system during the reaction (Fig. 4.2). A lower sulfate half-ester group content (0.29 ± 0.01 wt% versus 1.09 ± 0.01 wt%) increased the reaction rate coefficient by approximately 150%. The reaction rate for the oxidation of CNCs (k'_1) was $1.98 \cdot 10^{-5} \pm 1 \cdot 10^{-6}$ 1/s; whereas that for the oxidation of DeCNCs (k'_2) was $5.09 \cdot 10^{-5} \pm 4 \cdot 10^{-6}$ 1/s. The R-square values of the fittings indicated that both regressions were acceptable. The higher reaction rate of DeCNCs compared to CNCs suggests that sulfate half-ester groups hinder access to reactive sites on the cellulose molecule and retard the periodate oxidation process. It should be noted that CNCs were used in the Na^+ form because the addition of sodium periodate introduced a significant amount of sodium into the system, making the sodium content in CNCs negligible in comparison to that from periodate^{2,3,4}.

4.2.2 Effect of the sulfate half-ester group on CNC modification using LPMO9

The effect of sulfate half-ester groups on the modification of CNCs using LPMO9 was explored by studying both soluble and solid products of the reaction. Soluble products include molecules or fragments that are released into solution

²Ionic strength due to sodium cation present in the CNCs: 0.03 mM

³Ionic strength due to sodium cation present in the DeCNCs: 0.01 mM

⁴Ionic strength due to sodium metaperiodate (only Na^+ ions): 49.09 mM.

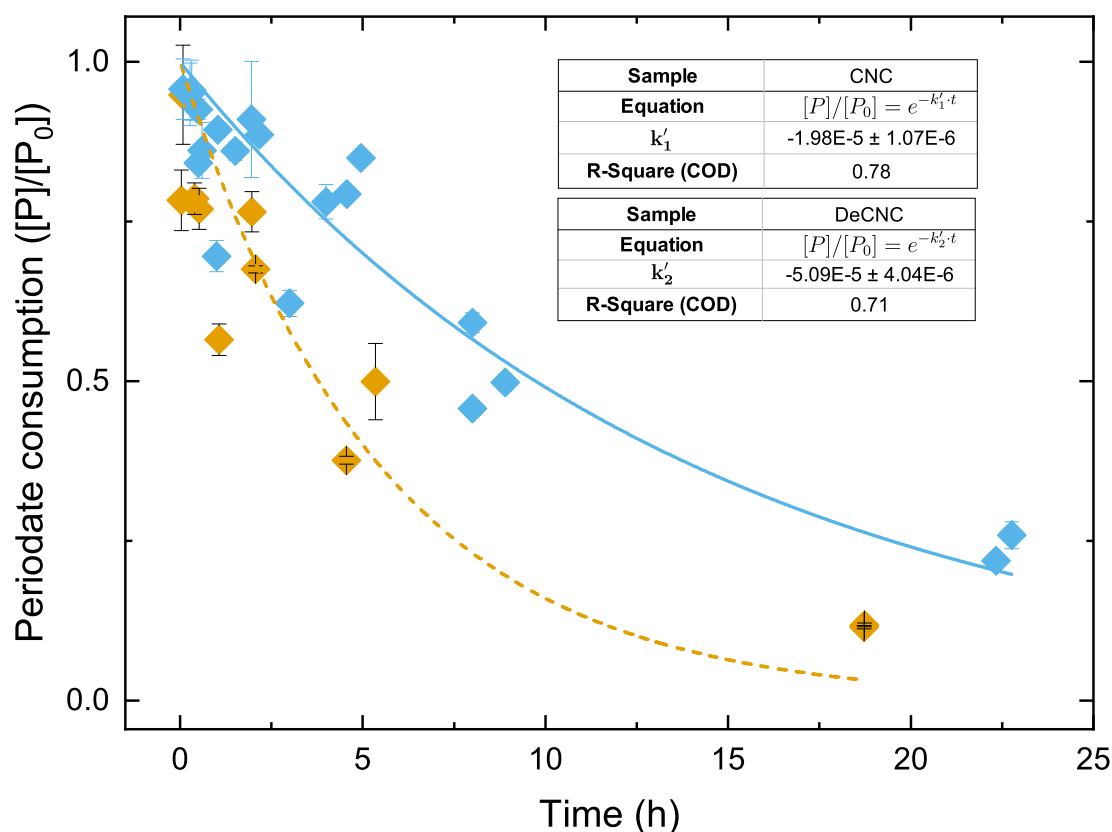


Figure 4.2: Consumption of periodate during the oxidation of CNCs and DeCNCs. The cyan diamonds and solid line represent the CNC data and fitting, while the orange diamonds and dashed line represent the DeCNC data and fitting. The equations contain fitted values for reaction rate constants denoted as k'_1 and k'_2 , respectively. The R-square value, which measures the goodness of fit of the regression, was used for statistical analysis. These curves represent three oxidation series for each CNC grade. Even though some points are not shown in the graph for clarity, they were included in the fitting and statistical analysis.

during enzymatic or chemical modification of CNCs. These products can provide valuable information on the specific sites and types of modifications that have occurred on cellulose chains, as well as their solubility and reactivity in different environments. However, it is important to note that not all modified chains will necessarily be released into solution.

Examination of the soluble products revealed that LPMO9 was active on both CNCs and DeCNCs, producing oxidized cellooligosaccharides with a degree of polymerization of 2 to 6 (Glc_{2ox}-6_{ox}). Quantification of the Glc_{2ox}-6_{ox} peak areas of each reaction revealed that LPMO9 released 38% more oxidized cellooligosac-

charides from DeCNCs than from CNCs (Paper III, Fig. 2). This discrepancy indicates a higher amount of soluble products, and thus more activity⁵, on DeCNC substrates compared to CNCs.

Solid products include modified CNCs that remain insoluble after enzymatic or chemical treatment. After 6 h of LPMO9 treatment, the carboxyl group content of CNCs augmented to 0.22 ± 0.01 mmol/g, a 10% increase over the NR control (0.20 ± 0.00 mmol/g) (Fig. 4.3). However, the carboxyl group content of DeCNCs was not statistically different from the NR control at approximately remained approximately 0.06 mmol/g (Paper III, Fig. 3). Notably, the control suspensions exhibited some residual carboxyl groups [143]. These findings are in contrast with the prevailing literature; while some studies observed an increase in carbonyl and carboxyl groups in the presence of sulfate half-ester groups, others report inhibitory effects by the sulfate half-ester groups [99, 144, 101].

On the one hand, if only the soluble fraction had been analyzed, DeCNCs would have appeared as more prone to modification owing to the higher content of soluble products in DeCNCs compared to CNCs. On the other hand, analysis of the solid fraction alone would have revealed no increase in carboxylation for DeCNCs but a 10% increase for CNCs. This observation would have led us to conclude that LPMO9 was not active in DeCNCs and the LPMO9 catalysis is enhanced at higher sulfate half-ester group content. By examining both fractions, we could conclude that LPMO9 was more active on DeCNCs, with the modified chains being released mainly as soluble products; whereas in the case of CNCs, the modification occurred mainly on the solid substrate.

The preference of LPMO9 for DeCNCs can be explained by the fact that the sulfate half-ester groups hinder, both the binding and hydrolysis of enzyme and CBM adsorption on the solid substrate. The enzyme operates in close proximity to the CBM. Consequently, the restricted adsorption of the CBM and the limited action of the enzyme near the sulfate half-ester groups reduce the probability that the same cellulose chain is cut multiple times a few AGUs apart [91]. The proximity of cut sites would enable the release of short soluble chains. Therefore, the absence of charged sulfate half-ester groups that could interfere with enzyme

⁵Note that activity is considered as hydrolysis and solubilization of cellulose, not the total modification.

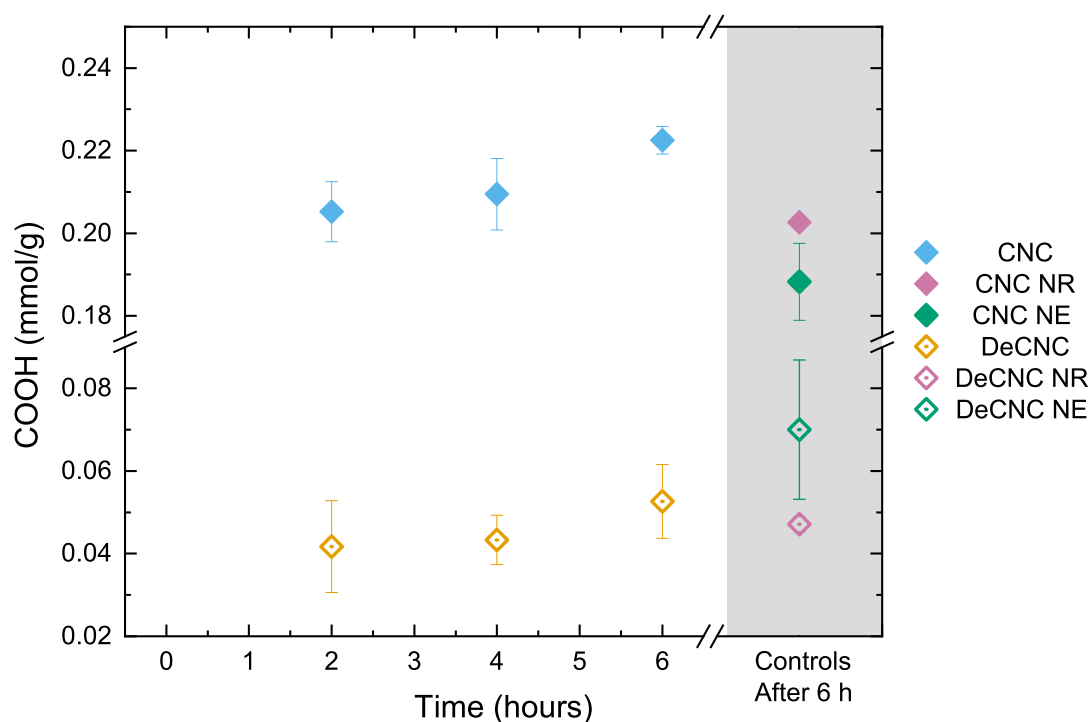


Figure 4.3: Carboxyl group content over reaction time for CNCs (filled diamonds) and DeCNCs (empty diamonds) as determined by conductometric titration. Values represent the average of at least three repetitions. Controls not subjected to treatment with reductant (NR) or LPMO9 (NE) for 6 h are also shown.

binding to DeCNCs, led to more extensive modification of cellulose chains, which were released into solution. Instead of hindering modification via LPMO9 or other oxidation reactions, as shown for periodate oxidation (Section 4.2.1), the above findings indicate that the charged sulfate half-ester groups played a crucial role in retaining the oxidized chains. In fact, charged sulfate half-ester groups appeared to facilitate rather than prevent modification by LPMO9, leading to successful carboxylation of CNCs.

Retrospectively, it would have been interesting to have studied the soluble product of periodate oxidation, and compared the amount of solubilized products based on CNC grades and the length of those chains. Such an approach would have allowed us to evaluate the degradation and fragmentation of CNCs

by periodate oxidation. The hydroxylamine assay revealed that the DO of insoluble products was lower (8% for CNCs and 15% for DeCNCs) than that determined by UV absorption at high DOs (Paper I, Figure 4.). That is because the latter quantifies the consumption of the reagent and, hence, does not discriminate between soluble and solid reaction products. This suggests that oxidized cellulose chains of less than 12-14 kDa were removed during dialysis.

4.2.3 Degradation of CNCs during oxidation

Given that oxidation sought to modify the surface properties of CNCs, it was important to examine if periodate or LPMO9 oxidation caused the degradation of CNCs. CNCs, deposited onto a silicon wafer, were subjected to Atomic Force Microscopy (AFM) for visualization and diameter determination (Paper I, Fig. 7). Meanwhile, their crystallinity was assessed by XRD (Paper I, Fig. 8). Periodate oxidation lowered the diameter of CNCs. The average diameter of a CNC was 4.1 ± 1.0 nm, while the average diameter of a DAC nanocrystal with a DO of 57% was 3.0 ± 0.8 nm, suggesting that cellulose chains peeled off. In contrast, DeCNCs increased in diameter, expanding from 4.0 ± 1.5 nm to 4.5 ± 1.0 nm at 53% DO. Even though the difference was not statistically significant from the non-modified grades, it could be attributed to the aggregation of crystals in the suspension. The crystallinity of DeCNCs was higher than that of CNCs, possibly due to removal of less organized cellulose chains by the acid used in the desulfation process. However, periodate oxidation decreased the crystallinity by 35% for both DAC and DeDAC compared to their non-modified grades.

Following oxidation by LPMO9, the average CNC diameter remained consistent at 3.7 ± 0.9 nm, which included both control and post-LPMO9 measurements (Paper III, Fig. 7). In the case of DeCNCs, desulfation caused the average diameter to augment to 5.7 ± 1.5 nm. This was attributed to DeCNC nanoparticle aggregation, which made it difficult to discern individual particles. After modification by LPMO9, the diameter of DeCNCs were approximately the same. Similarly, the crystallinity, averaging at 82% for CNCs and 87% for DeCNCs across control and post-LPMO9 oxidation, showed that enzymatic oxidation did not significantly alter their structure (Paper III, Supporting Information Fig. 8).

In conclusion, periodate oxidation of CNCs resulted in a smaller CNC diameter, indicating peeling or degradation; whereas LPMO9 did not reduce the diameter of CNCs, suggesting that no peeling occurred. In both modifications, the diameter of DeCNC increased due to aggregation, which hindered any estimation of degradation. The presence of soluble products following LPMO9 oxidation or decrease of crystallinity upon periodate oxidation suggested some extent of degradation. The decrease in crystallinity observed for both grades due to periodate oxidation resulted from the destruction of their ordered packing. Kim et al. [145] suggested that the modification occurred in localized regions or clusters, which disrupted the ordered structure of CNCs. In contrast, oxidation by LPMO9 did not alter CNC crystallinity. Hence, periodate oxidation may degrade or result in greater peeling of the CNC surface compared to what is obtained with LPMO9 in the conditions studied. As shown here, it remains a challenge to achieve extensive surface functionalization without compromising CNCs through their degradation.

4.2.4 Degree of surface substitution in CNCs

DSS was established in this thesis as a quantitative measure of the extent to which a particular functional group was present on the surface of CNCs. Both chemical and enzymatic modification methods introduce functional groups on the surface of CNCs. DSS was determined for two different functionalities: sulfate half-ester group DSS_S and oxidized cellulose DSS_{ox} . The oxidized cellulose represented by dialdehyde cellulose resulting from periodate oxidation and carboxylated cellulose resulting from modification using LPMO9. The distribution of functional groups on the surface of CNCs is summarized in Table 4.1.

After periodate oxidation, the content of sulfate half-ester groups was substantially reduced, from 15% to 6% in the case of CNCs, and from 3.8 % to 2.3 % in the case of DeCNCs. The decrease in DSS_S for both CNCs and DeCNCs indicated that sulfate half-ester groups peeled off during oxidation with periodate. Hence, this treatment was sufficiently harsh to hydrolyze cellulose chains close to the sulfate half-ester groups. This, in turn, may affect their colloidal and electrochemical properties, which is crucial for further chemical modifications or network formation. Furthermore, the CNC and DeCNC surfaces were fully dec-

Table 4.1: Distribution of functional groups on the surface of CNCs as a result of oxidation using periodate or LPMO9. The first column indicates the type of modification (periodate or LPMO9), and the second column identifies the system. R_{sur} : Percentage of AGUs on the surface compared to total AGUs. S : number of AGUs for each sulfate half-ester group. DO: degree of oxidation. DSS_S : Percentage of AGUs with a sulfate half-ester group on the surface. DSS_{DO} : degree of oxidation. DSS_{ox} : Percentage of surface AGUs oxidized either by periodate or LPMO9. DSS_U : degree of surface needed to achieve such an amount of dialdehyde cellulose on the surface.

Modification	Sample	R_{sur} (%)	S	DO (%)	DSS_S (%)	DSS_{ox} (%)	DSS_U (%)
Periodate	CNC	38	18	0	15.0	0	-
	DeCNC	38	69	0	3.8	0	-
	DAC	56	30	57	6.0	102	203
	DeDAC	53	84	53	2.3	100	200
LPMO9	CNC NR	44	28	3.1	8.1	7.0	-
	DeCNC NR	32	314	0.8	1.0	2.5	-
	CNC 6h	44	28	3.6	8.1	8.2	-
	DeCNC 6h	37	314	0.9	0.9	2.3	-

orated with aldehyde groups following periodate oxidation. Thus, taking into account the availability of C2 and C3, the DSS_U value shows that it would have taken twice the surface to achieve the observed degree of superficial aldehyde groups. Therefore, periodate oxidation was not limited to the surface, but occurred also elsewhere. The DO values reported in this study were determined using UV absorbance, which accounted for both soluble and solid modified products. Nevertheless, the widespread occurrence of the reaction would have been confirmed even if DO values were derived from the hydroxylamine assay for solid products, as the difference in DO between both methods was less than 15%.

After 6 h of oxidation by LPMO9, the calculated degree of sulfate half-ester groups on the CNC surface remained constant. Based on available surface groups, 3.1% (CNC NR) and 0.8% (DeCNC NR) presented a sulfate half-ester group and these values were unaffected by LPMO9. Following LPMO9 usage, the percent-

age of AGUs with a carboxyl group increased by 1.2% to 8.2% of AGUs when CNCs were used. Notably, this increase was not detected when DeCNCs were used, which was already discussed in Section 4.2.2. Overall, both periodate oxidation and LPMO9 oxidation (only in the case of sulfated CNC) successfully added new functional groups to the CNCs' surface, and the degree and distribution of these functional groups were influenced by the content of sulfate half-ester groups. Among the two treatments, periodate oxidation was more efficient at introducing surface oxidative modifications, but it also led to a decrease in sulfate half-ester groups likely due to the peeling effect observed in Section 4.2.3.

4.2.5 Colloidal stability of modified CNCs

Sulfate half-ester groups significantly affect the colloidal stability of CNCs, providing electrostatic repulsion between particles. As a result, the particles remain dispersed for a long period of time. Periodate oxidation was found to lower the zeta potential of both CNCs and DeCNCs, leading to a decrease in colloidal stability. Prior to periodate oxidation, the zeta potential values for CNCs and DeCNCs were -28 ± 5 and $-19 \text{ mV} \pm 2$, respectively (Paper I, Fig. 6). When the degree of oxidation (DO) reached approximately 50%, the zeta potential of both grades decreased to approximately $-5 \text{ mV} \pm 2$. In contrast, oxidation by LPMO9 did not significantly alter the zeta potential of CNCs or DeCNCs. The average zeta potential for CNCs, considering both LPMO9-treated samples and controls, was $-37 \pm 2 \text{ mV}$ (Paper IV, Fig. 5). Similarly, for DeCNCs, the average zeta potential following LPMO9 treatment along with controls was $-30 \pm 1 \text{ mV}$. Evidently, the minor variations within the error range suggest that the LPMO9 treatment did not considerably alter the zeta potential of either CNCs or DeCNCs. It was also clear that desulfation generally led to a reduction in sulfate half-ester groups, and consequently, the charge content on the CNCs and its colloidal stability (Sections 4.2.3 and 4.2.4).

Aldehyde groups introduced by periodate oxidation could be used to crosslink CNCs within or across chains [69]. The formation of inter-particle hemiacetal bonds could lead to aggregation of the particles, affecting their colloidal stability and possibly explaining the observed low zeta potential values (-5 mV). In contrast, LPMO9 modification introduced carboxyl groups, which provided ad-

ditional negative charge and enhanced electrostatic repulsion between particles, thereby also resulting in a low zeta potential. This implies that periodate oxidation led to a decrease in DSS_s (Section 4.2.4) and thus a decrease on the colloidal stability of CNCs, while oxidation by LPMO9 resulted in no significant alteration of colloidal stability.

4.3 Network formation using CNCs

One of the intriguing properties of CNCs is their ability to act as building blocks and create a three-dimensional network in water. In this section, I will discuss the impact of trivalent cations and TBA on the rheology of CNC suspensions, as well as the means of enhancing the gel strength and thermal stability of gelatine gels by adding CNCs.

4.3.1 Effect of trivalent cations and TBA on the rheology of CNC networks

Steady shear and oscillatory shear measurements were conducted to investigate the viscosity and elasticity of CNC suspensions containing Eu³⁺, Gd³⁺, and Dy³⁺ cations, as well as the effect of TBA as co-dispersant in CNC-water suspensions. CNCs used in both systems were derived from the same batch, with an average sulfur content of 0.72 ± 0.20 wt%, which corresponded to one sulfate half-ester group for every 27 to 29 AGUs.

The inclusion of trivalent cations was characterized by elemental analysis. The observed lanthanide to sulfur atomic ratio of Eu³⁺ to S was 0.43, Gd³⁺ to S was 0.27, and Dy³⁺ to S was 0.14. Although these trivalent cations were initially added in a 1:1 molar ratio to the sulfate half-ester groups, the inherent dynamic system, affinity of the trivalent cations, and dialysis resulted in the observed atomic ratio. The CNCs (Na⁺ as a cation), had an atomic ratio of Na⁺ to S of 0.83. The CNC suspensions with trivalent cations exhibited shear thinning behavior; whereas CNCs exhibited a shear thinning behavior with a zero-shear Newtonian plateau at low shear rates (Fig. 4.4a). The suspensions with trivalent cations showed an increase in viscosity of up to three orders of magnitude relative to CNCs at low shear rates. Moreover, polarized optical microscopy

revealed the presence of structures, especially in Dy^{3+} and Gd^{3+} suspensions (Paper V, Fig. S5). Oscillatory measurements revealed the presence of a gel network in the suspensions with trivalent cations (Fig. 4.4b-c). The CNC suspensions with trivalent cations were above their respective gel points at this CNC and lanthanide concentration, with $G' > G''$ across the entire angular frequency range investigated. In contrast, the CNC suspension displayed a predominantly viscous behavior ($G'' > G'$). Interestingly, the rheological properties were not dependent on the lanthanide to sulfur atomic ratio. The viscosity at 1 1/s was higher for Dy^{3+} -CNC, followed by Gd^{3+} -CNC, and then Eu^{3+} -CNC; whereas G' within the viscoelastic region was highest for Gd^{3+} -CNC, followed by Dy^{3+} -CNC, and lastly Eu^{3+} -CNC. Thus, given that all lanthanides were trivalent, these rheological properties were influenced by factors other than the atomic ratios and charge.

The addition of TBA as a co-dispersant was studied by rheological measurements. For suspensions containing 2 wt% CNC, the addition of 19 wt% TBA did not change the isotropic behavior from that of a typical CNC suspension with low CNC content (Fig. 4.5a) [119]. Instead, a single shear-thinning region was achieved at 50 wt% TBA; while at TBA concentrations above 60 wt%, the suspension's phases separated and thus not shown. In the linear region of the oscillatory shear strain sweep, a comparison of suspensions containing 2 and 3 wt% CNCs revealed that the G' increased with the addition of TBA, whereas $\tan(\delta)$ decreased following the addition of TBA indicating a more solid-like or elastic behavior (Fig. 4.5b). In the oscillatory frequency sweep (Fig. 4.5c), the CNC-water suspensions with 3 wt% displayed a predominantly viscous behavior ($G'' > G'$). Addition of 19 wt% TBA to a 2 wt% CNC suspension exhibited crossover of $G' > G''$ at lower frequencies compared to the 3 wt% CNC suspension with no TBA addition. At higher frequencies, the suspension was characterized by $G' \simeq G''$. The behavior exhibited by the suspension with 2 wt% CNC and 19 wt% TBA indicated structuring of TBA even at low concentrations. The addition of 56 and 50 wt% TBA to 2 and 3% wt CNC suspensions resulted in a gel network with $G' > G''$ across the entire angular frequency range investigated, confirming the role of TBA as structuring agent for CNC-water suspensions. The increase in shear viscosity by addition of TBA reached a maximum with 50 wt% (Paper IV, Fig. 5). da Silva et al. [42] investigated the gelation of carboxylated

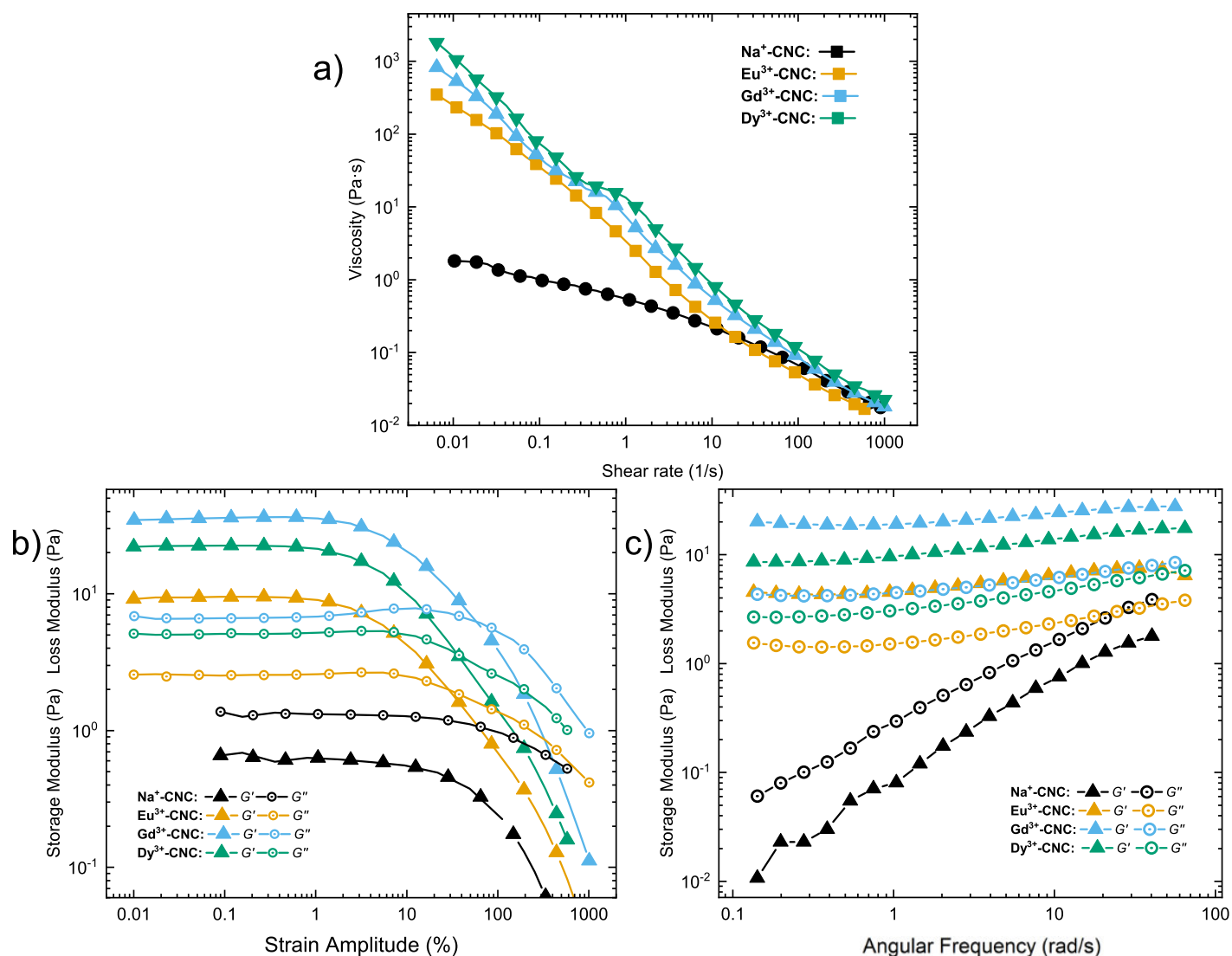


Figure 4.4: (a) Shear viscosity, (b) amplitude sweep, and (c) frequency sweep measurements of 2 wt% CNC suspensions with trivalent cations. Shear viscosity was measured at shear rates ranging from 0.01 to 1000 1/s. Amplitude sweep measurements were conducted at 6.28 rad/s and strain amplitude at 0.01% to 1000%. Frequency sweep measurements were conducted at 0.1 to 70 rad/s at a constant strain amplitude of 0.25%. G' is represented by triangles and G'' by circles. Na⁺-CNC is shown in black, Eu³⁺-CNC in orange, Gd³⁺-CNC in cyan, and Dy³⁺-CNC in green. The atomic ratio of Eu³⁺ to S was 0.43, Gd³⁺ to S was 0.27, Dy³⁺ to S was 0.14, and Na⁺ to S was 0.83.

CNF in water/alcohol mixtures, finding that low molecular alcohols, when used as co-dispersants, increased the viscosity and gel strength of CNF dispersions, similar to the effects of TBA in CNC suspensions. They attributed this behavior

4. Results and Discussion

to the reduced solvation of carboxyl groups and increased hydrophobic interactions between CNFs. Furthermore, da Silva et al. [42] observed phase separation of nanocelluloses at alcohol concentrations exceeding 50 wt

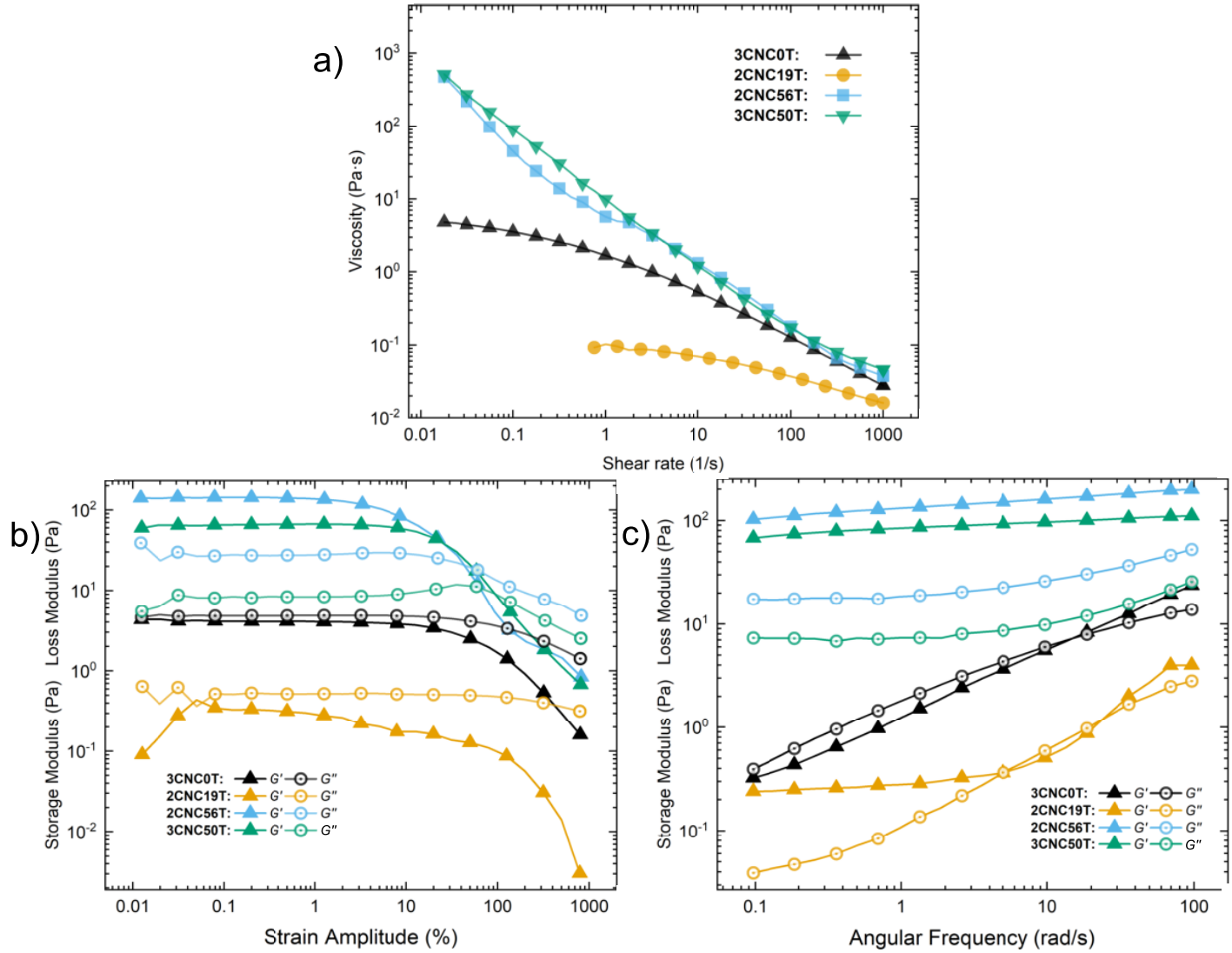


Figure 4.5: (a) Shear viscosity, (b) amplitude sweep, and (c) frequency sweep measurements of 2 wt% CNC-water suspensions containing either 19 wt% TBA (orange) or 56 wt% TBA (cyan), and a 3 wt% suspension containing 50 wt% TBA (green). Shear viscosity measurements were conducted at 0.01 to 1000 1/s. Amplitude sweep measurements were conducted at 6.28 rad/s, within a strain amplitude range of 0.01% to 1000%. Frequency sweep measurements were conducted at 0.1 to 100 rad/s, with a strain amplitude of 0.3%. G' is represented by triangles and G'' by circles.

The mechanism responsible for an increase in viscosity of CNC suspensions

harboring lanthanide ions involves the interaction between trivalent cations and sulfate half-ester groups on CNCs. Metal cations, with their large ionic radii and low solubility in water, may coordinate to several adjacent sulfate half-ester groups on CNC particles [120]. However, charge screening was suggested as a main driver of gel networks. Charge screening occurs when cations neutralize the negative charges of sulfate half-ester groups on the CNC surface, reducing the electrostatic repulsion between particles. Here, charge screening promoted the formation of these structures, resulting in gels whose viscosity was significantly higher compared to that of suspension containing Na^+ .

When TBA is used as a co-dispersant for CNC suspensions, the increase in viscosity arises from altered CNC-CNC interactions and structuring in the water-TBA system. As the TBA concentration increases, the balance between repulsive and attractive forces in the CNC suspension shifts, allowing the formation of interconnected networks and, consequently, gels (Fig. 4.5c).

In summary, the addition of trivalent cations or TBA into CNC suspensions increased their viscosity by favoring the formation of an interconnected network. Although both systems showed similarities, such as triggered gelation or shear thinning behavior, the mechanisms responsible for the increase in viscosity differed: trivalent cations interacted with sulfate half-ester groups on CNCs; whereas addition of TBA as a co-dispersant modified the CNC-CNC interactions and structured the CNC-water system.

4.3.2 Enhancing the rheological and thermal stability of gelatine emulsion gels using CNCs

In this section, I investigated the possibility of enhancing the properties of CNC-based materials by incorporating CNCs and DACs in a gelatine emulsion. This strategy sought to counteract the instability of gelatine at higher temperatures than approximately 35 °C, where it tends to melt. Unlike with systems containing trivalent cations or TBA, this approach focused on the role of CNCs as physical or chemical crosslinkers in a gelatine network. The challenge was to improve the thermal stability of the gelatine emulsion gel in which oil droplets were dispersed in the gelatine-water matrix and could withstand heating.

To achieve this goal, three different gelatine-based systems were developed:

emulsions reinforced by gelatine only, emulsions reinforced by gelatine and CNCs, and emulsions reinforced by gelatine and DAC (DO 14%). DAC was obtained by subjecting CNCs to periodate oxidation. Shear rheology was employed to measure the moduli and the temperatures at which gels set and melted, determined at the crossover ($G' = G''$) in the cooling and heating curves respectively. The gelatine emulsion gel exhibited typical gelatine behavior, setting at 30 °C and melting at 38 °C upon reheating (Fig. 4.6a). In contrast, CNC-gelatine and DAC-gelatine reinforced emulsions did not show clear setting or melting transitions, displaying instead a predominantly elastic behavior with $G' > G''$ (Fig. 4.6b-c). However, CNC-reinforced emulsions exhibited moduli of 80-100 Pa at temperatures of 40-60 °C, and 2000-3000 Pa at temperatures of 10-25 °C. Thus, the decrease upon heating by approximately 95%. This shows a very large change in the rheological behavior of the CNC-reinforced emulsions due to temperature.

The reinforcement of gelatine emulsion gels by CNC grades can be explained by the formation of a network structure between the gelatine and the CNC grades. Addition of CNCs to the gelatine emulsion gels resulted in a similar effect on the melting point as that observed with DAC, even though CNCs were not expected to chemically interact with gelatine. This phenomenon could be explained by a larger density of hydrogen bonds and electrostatic attraction between positively charged gelatine and negatively charged CNCs or DAC that promoted structural stability [146]. The latter then enhanced resistance to deformation and resulted in higher elastic moduli. Alternatively, DAC's lower colloidal stability may have led to a heterogeneous network highlighting the importance of colloidal stability for the use of CNCs. In either case, the presence of CNCs in the emulsion appears to have a comparable impact on the strength of the gel as the intended chemical crosslinking with DAC, yielding a stable emulsion with improved strength and thermal stability.

A comparison of the systems containing CNCs with trivalent cations, TBA as co-dispersant, and CNCs as reinforcement of gelatine emulsion gels highlights the versatility of CNCs in tailoring the properties of various materials. In the trivalent cation and TBA systems, the interaction with CNCs led to the formation of interconnected networks. In contrast, in the gelatine system, CNCs reinforced the gelatin network enhancing the thermal stability and gel strength.

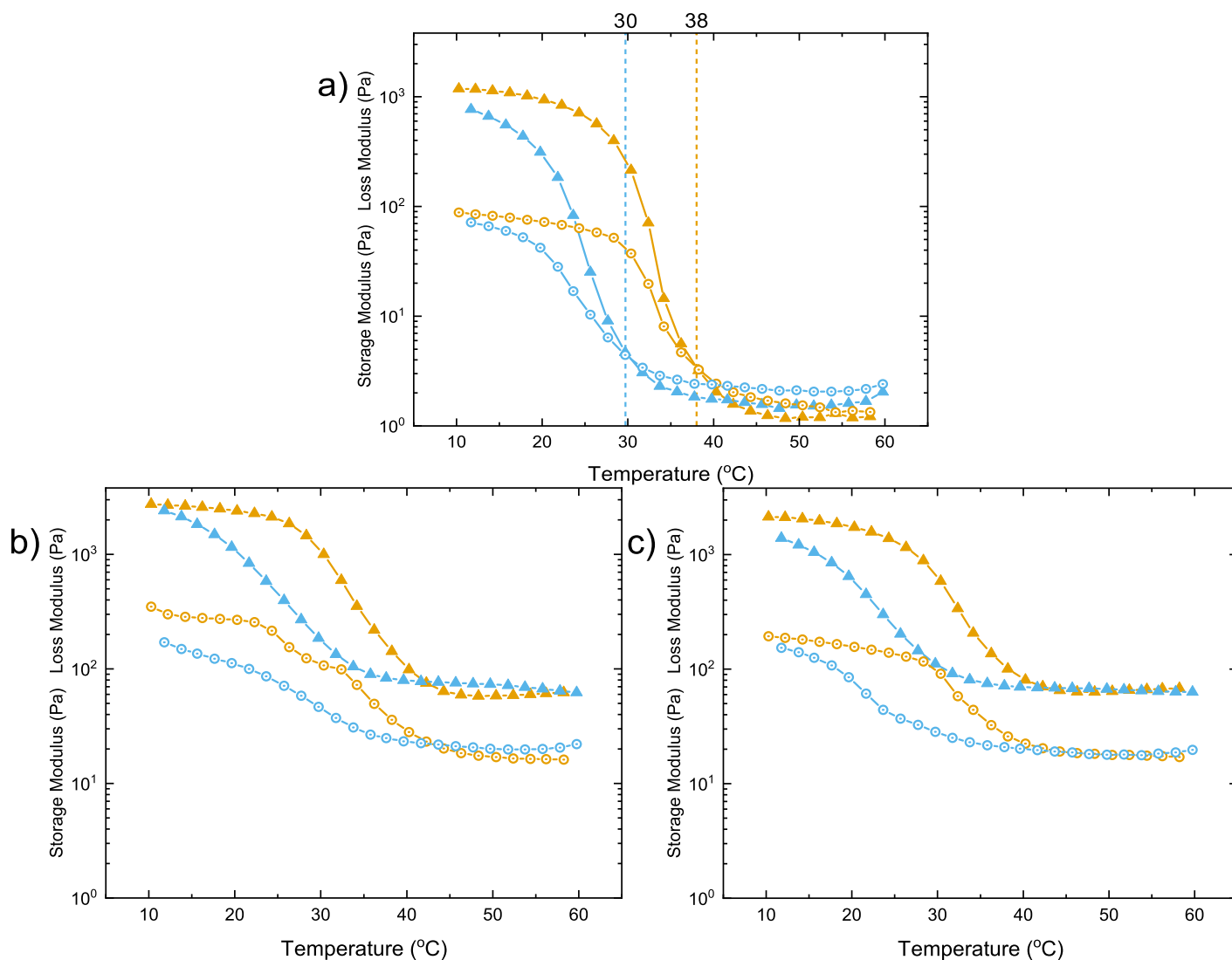


Figure 4.6: Representation of G' (triangles) and G'' (circles) as temperature-dependent functions for (a) gelatine emulsion gel, (b) CNC-gelatine reinforced emulsion gel, and (c) DAC-gelatine reinforced emulsion gel. The emulsions were initially maintained at 60 $^{\circ}\text{C}$ and subsequently subjected to cooling (in cyan) and heating (in orange). Dashed vertical lines denote the gel's setting and melting temperatures. Measurements were carried out at a shear rate of 1 $1/\text{s}$ and a strain of 0.5%.

5

Conclusion

The aim of this thesis was to determine the role of sulfate half-ester groups in the oxidative modification of cellulose nanocrystals, as well as subsequent formation of networks in combination with trivalent cations and TBA. The thesis has revealed that sulfate half-ester groups exert varying effects on oxidative modifications. Such groups are negatively charged and bulky, which hinders oxidation reactions and influences the properties of the resulting products. Specifically, sulfate half-ester groups reduce the kinetics of the periodate oxidation reaction. Periodate oxidation lowers the sulfate content and colloidal stability, while at the same time triggering degradation and peeling of CNCs. Sulfate half-ester groups prevent the peeling of cellulose chains during enzymatic oxidation by LPMO9, promoting instead the retention of carboxy groups on the surface. Using LPMO9 to oxidize CNCs preserved the sulfur content and colloidal stability, without any unwanted peeling. Importantly, the study highlights the need for analyzing both solid and soluble products to avoid incorrect conclusions. This thesis has elucidated the process of obtaining CNCs with both sulfate half-ester groups and either aldehydes or carboxyl groups through oxidative modifications. CNCs and DAC reinforced a three-dimensional gelatine network through physical and/or chemical crosslinking, thereby increasing gel strength and temperature stability of such gelatine emulsion gels.

The addition of trivalent cations was shown to screen the sulfate half-ester groups present on the CNCs, leading to gel networks with increased viscosity and elasticity. Used as a co-dispersant, TBA was capable of modifying CNC structuring, leading to higher shear viscosity, and triggering gelation at lower CNC concentrations than without the structuring agent.

The research faced analytical challenges, particularly related to the determination of functional groups on CNCs and the degradation of oxidized CNCs.

5. Conclusion

These issues highlight the complex nature of nanocellulose research.

Nevertheless, this thesis has contributed to the development of nanotechnology built on wood nanoscience. It has demonstrated the importance of characterizing sulfate half-ester groups and of functional groups into account when modifying and engineering materials. It has also provided fundamental information about the chemical characteristics of both oxidation reactions and the resulting product, which are crucial for utilizing oxidized CNCs. Finally, the research described herein demonstrates the feasibility of using LPMOs as a potentially more eco-friendly method of functionalizing CNCs compared to traditional chemical pathways.

6

Outlook

Despite the progress made in understanding the role of sulfate half-ester groups. The research carried out here underlines the critical role of charged groups in the efficiency of the modification and their influence on assembly in suspensions. It is therefore implied that the distribution of these charged groups likely contributes significantly to these phenomena.

The complex nature of enzymatic systems and their interactions with cellulose make it challenging to understand their mode of action and binding. While this thesis has contributed to acknowledging the importance of sulfate half-ester groups in retaining specific CNC functionalities, there are still knowledge gaps concerning enzyme binding mechanisms, the role of CBMs, and activity on CNCs with different sulfur half-ester content. Exploring the positional hydrolysis of cellulose by LPMOs could help determine whether these enzymes hydrolyze randomly or in a defined manner, which could improve the surface-specificity of the enzymatic modification approach.

The extent of coordination of trivalent cations to sulfate half-ester groups is another open question. Elucidating the mechanism of coordination would provide valuable insights on the valorization of lanthanide-containing CNC suspensions in optical applications.

Alcohols can modify the structure of nanoparticles. This thesis has demonstrated that TBA can guide CNC network formation, but the position of CNCs in the TBA-water system and the influence of hydrophobic interactions remain unclear. Investigating the role of hydrophobic effects in network formation and the impact of different alcohols on CNC network forms could usher in a new approach for structuring nanoparticles into materials. Addressing these research questions and hypotheses will sustain the expansion of the field of CNCs, leading to new discoveries, applications, and sustainable technologies.

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