Tailoring Cellulose Nanofibrils for Advanced Materials

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Doctoral Thesis
Stockholm 2014
AKADEMISK AVHANDLING
som med tillstånd av Kungliga Tekniska Högskolan i Stockholm framläggs till offentlig
granskning för avläggande av teknologie doktorsexamen den 21 November 2014, kl.
13.30 i sal F3, Lindstedtsvägen 26, KTH.
Fakultetsopponet: Prof. Kristiina Oksman från Luleå Tekniska Universitet.
Avhandlingen försvaras på engelska.

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TRITA CHE Report 2014:52
ISSN 1654-1081

In memoriam of Montse Boix,
who taught me what a polymer is.
ABSTRACT

Cellulose nanofibrils (CNFs) are nanoscale fibers of high aspect ratio that can be isolated from a wide variety of cellulosic sources, including wood and bacterial cellulose. With high strength despite of their low density, CNFs are a promising renewable building block for the preparation of nanostructured materials and composites. To fabricate CNF-based materials with improved inherent rheological and mechanical properties and additional new functionalities, it is essential to tailor the surface properties of individual CNFs. The surface structures control the interactions between CNFs and ultimately dictate the structure and macroscale properties of the bulk material. In this thesis we have demonstrated different approaches, ranging from non-covalent adsorption and covalent chemical modification to modification of cellulose biosynthesis, to tailor the structure and surface functionalities of CNFs for the fabrication of advanced materials. These materials possess enhanced properties such as water-redispersibility, water absorbency, dye adsorption capacity, antibacterial activity, and mechanical properties.

In Paper I, CNFs were modified via the irreversible adsorption of carboxymethyl cellulose (CMC). The adsorption of small amounts of CMC onto the surface of CNFs prevented agglomeration and co-crystallization of the nanofibrils upon drying, and allowed the recovery of rheological and mechanical properties after redispersion of dried CNF samples.

In Paper II, CNFs bearing permanent cationic charges were prepared through quaternization of wood pulp fibers followed by mechanical disintegration. The activation of the hydroxyl groups on pulp fibers by alkaline treatment was optimized prior to quaternization. This optimization resulted in individual CNFs with uniform width and tunable cationic charge densities. These cationic CNFs demonstrated ultrahigh water absorbency and high adsorption capacity for anionic dyes.

In Paper III, via a similar approach as in Paper II, CNFs bearing polyethylene glycol (PEG) were prepared by covalently grafting PEG to carboxylated pulp fibers prior to mechanical disintegration. CNFs with a high surface chain density of PEG and a uniform width were oriented to produce macroscopic ribbons simply by mechanical stretching of the CNF hydrogel network before drying. The uniform grafted thin monolayer of PEG on the surface of individual CNFs prevented the agglomeration of CNFs and facilitated their alignment upon mechanical stretching, thus resulted in ribbons with ultrahigh tensile strength and modulus. These optically transparent ribbons also demonstrated interesting biaxial light scattering behavior.

In Paper IV, bacterial cellulose (BC) was modified by the addition of chitin nanocrystals (ChNCs) into the growing culture medium of the bacteria Acetobacter aceti which secretes cellulose in the form of entangled nanofibers. This led to the in situ incorporation of ChNCs into the BC nanofibers network and resulted in BC/ChNC nanocomposites exhibiting bactericidal activity. Further, blending of BC nanofibers with ChNCs produced nanocomposite films with relatively lower tensile strength and modulus compared to the in situ cultivated ones. The bactericidal activity increased significantly with increasing amount of ChNCs for nanocomposites prepared by direct mixing of BC nanofibers and ChNCs.

In Paper V, CNFs were isolated from suspension-cultured wild-type (WT) and cellulose-binding module (CBM) transformed tobacco BY-2 (Nicotiana tabacum L. cv bright yellow) cells. Results from strong sulfuric acid hydrolysis indicated that CNFs from transgenic cells overexpressing CBM consisted of longer cellulose nanocrystals compared to CNFs from WT cells. Nanopapers prepared from CNFs of transgenic cells demonstrated significantly enhanced toughness compared to CNFs of WT cells.

Keywords: Cellulose nanofibrils; bacterial cellulose; surface modification; carboxymethyl cellulose; polyethylene glycol; chitin nanocrystals; bactericidal activity; nanofibrils orientation; water redispersibility; mechanical properties; dye removal.
SAMMANFATTNING

Nanofibriller från cellulosa (CNF) har en diameter på nanoskala, högt slankhetstal (längd/diameter) och kan isoleras från råvaror som t ex trä och bakteriecellulosa. CNF har hög hållfasthet i förhållande till sin densitet och betraktas som en lovande materialkomponent från förnyelsebar råvara, särskilt för framställning av nanostrukturerade material och kompositer.


I artikel I modifierades CNF genom irreversibel adsorption av karboxymetylcellulosa (CMC). Det förhindrade agglomerering och samkristallisering av CNF under torkning. Dessutom återskapades de reologiska och mekaniska egenskaperna hos kolloider och gjutna filmer efter återdispergering.

I artikel II framställdes CNF med katjonisk laddning genom kemisk behandling av massafibrer följt av mekanisk disintegrering. Hydroxylgrupperna på cellulosa aktiverades genom behandling med alkali. Det resulterade i CNF med homogen diameter, nära den för mikrofibriller, och en laddningsdensitet som kunde styras. Dessa katjoniska fibriller visade hög vattenadsorption och också hög kapacitet att adsorbera anioniska färgämnen.


I artikel IV modifierades bakteriecellulosa (BC) genom tillsats av nanokristaller från kitin (ChNCs). ChNC placerades i den växande odlingen av bakterien Acetobacter aceti, som utsöndrar cellulosa i form av bandstrukturer med nanodimensioner. Det innebar att ChNC blev inneslutna i det nätverk av BC som utsöndrades, så att man fick kompositer av BC/ChNC med baktericida egenskaper. Det visade sig dessutom att enkel mekanisk blandning av BC och ChNC resulterade i filmer som hade lägre E-modul och hållfasthet än de som producerades in-situ. De baktericida egenskaperna ökade signifikant med ökande mängd ChNC, för de nanokompositer som framställdes genom enkel mekanisk blandning.

I artikel V isolerades CNF från vildtyp (WT) cellsuspensionskulturer av tobak (Nicotiana tabacum L. cv bright yellow) och från kulturer transformerade med en kolhydratbindandemodul (CBM). Resultat erhållna efter stark svavelsyrahydrolys visade på att CNF från de transgena cellerna bestod av längre cellulosananokristaller i jämförelse med CNF från vildtypen. Nanopapper framställt av CNF från de transgena cellerna hade betydlig högre seghet i jämförelse med WT cellerna.
LIST OF PUBLICATIONS

This thesis is based in the following publications:

**Paper I.** Water redispersible cellulose nanofibrils adsorbed with carboxymethyl cellulose.
N. Butchosa and Q. Zhou.

**Paper II.** Surface quaternized cellulose nanofibrils with high water absorbency and adsorption capacity for anionic dyes.
A. Pei, N. Butchosa, L.A. Berglund and Q. Zhou.

**Paper III.** Transparent, Hazy, and Strong Macroscopic Ribbon of Oriented Cellulose Nanofibrils Bearing Poly(ethylene glycol).
Submitted.

**Paper IV.** Nanocomposites of bacterial cellulose nanofibers and chitin nanocrystals: fabrication, characterization and bactericidal activity.
Green Chemistry 15 (12), 3404-3413.

**Paper V.** Enhancing toughness of cellulose nanofibrils through the expression of cellulose-binding modules in plant primary cell wall.
N. Butchosa, F. Leijon, V. Bulone and Q. Zhou.
Submitted.

The contributions of the author of this thesis to the above listed publications are:

**Paper I:** All of the experimental work and all of the manuscript preparation.
**Paper II:** Part of the experimental work and part of the manuscript preparation.
**Paper III:** Part of the experimental work and part of the manuscript preparation.
**Paper IV:** All of the experimental work except cell cultivation, and all of the manuscript preparation.
**Paper V:** All of the experimental work except cell cultivation, and all of the manuscript preparation.
Other relevant publications not included in this thesis:

**Nanopaper membranes from chitin–protein composite nanofibers - structure and mechanical properties.**

**Nanostructured membranes based on native chitin nanofibers prepared by mild process.**

**Glycan-Functionalized Fluorescent Chitin Nanocrystals for Biorecognition Applications.**
LIST OF ABBREVIATIONS

AFM: atomic force microscopy
AGU: anhydroglucose unit
BC: bacterial cellulose
CBMs: cellulose-binding modules
CBM3: cellulose-binding module from family 3
cfu: colony forming unit
ChNCs: chitin nanocrystals
CDTA: 1,2-Diaminocyclohexanetetraacetic acid
CMC: carboxymethyl cellulose
CNC: cellulose nanocrystal
CNF: cellulose nanofibril
$^{13}$C-NMR: 13 carbon-nuclear magnetic resonance
DA: degree of acetylation
DP: degree of polymerization
DS: degree of substitution
E. coli: Escherichia coli
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
FE-SEM: field emission scanning electron microscopy
FT-IR: Fourier-transformed infrared spectroscopy
HS: Hestrin-Scheramm
LB: Luria broth
NHS: n-hyrdroxysuccinimide sulfonate
PEG: polyethylene glycol
PEG-NH$_2$: methoxy polyethylene glycol amine
STEM: scanning electron microscopy in transmission mode
SDS: sodium dodecyl sulfate
TEM: transmission electron microscopy
TEMPO: teramethylpiperidine-1-oxyl radical
TGA: thermogravimetric analysis
WAXD: wide angle x-ray diffraction
WT: wild-type
XRD: x-ray diffraction
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1. INTRODUCTION

1.1. Objectives

The objective of this thesis was the modification of cellulose nanofibrils (CNFs) to improve their inherent outstanding properties and to achieve new functionalities. The scientific aim of this work was to study how different modifications influenced the final morphology, processability and properties of the resulting CNFs and CNF-based materials. Moreover, it was our goal to achieve optimized modification conditions, and to develop a deep understanding of the structure-properties relationship in the materials. Furthermore, it was also our target to promote new fields of application for CNFs and CNF-based materials.

1.2. Cellulose and cellulose nanoparticles

Cellulose is a naturally occurring polymer produced by a wide variety of organisms. Plants, algae, some bacteria, and a few animals synthesize cellulose. Owing to its numerous and abundant sources, cellulose is the most abundant natural polymer on Earth. The molecular structure of cellulose was discovered by Anselm Payne in 1836. As shown in Figure 1, cellulose is a linear chain of β-1,4 linked D-glucose residues. The degree of polymerization (DP) of cellulose is typically of several hundreds to a few thousands. Due to its extended chain and its numerous hydroxyl groups, cellulose tends to pack in a crystalline manner.

The most common crystallographic allomorphs of cellulose are cellulose I and cellulose II.\textsuperscript{1,2} Naturally occurring cellulose exhibits the cellulose I allomorph, with
parallel packing of the cellulose chains. Cellulose II, also known as regenerated cellulose, is produced by the mercerization of cellulose I. The mercerization process leads to a more thermodynamically stable conformation, i.e. an antiparallel arrangement of the cellulose chains. The crystalline packing of cellulose, with numerous intramolecular and intermolecular hydrogen bonds, is the reason why cellulose is very hard to dissolve in both polar and non-polar solvents. Furthermore, the cellulose structure is the cause of its outstanding physical properties, such as high tensile strength and elastic modulus, and low density and thermal conductivity. The properties of cellulose compared to some reinforcement materials are summarized in Table 1. With very similar densities, crystalline cellulose shows a better mechanical performance than Kevlar. Even though it is almost 5 times lighter, crystalline cellulose possesses a higher tensile strength and a similar elastic modulus compared to steel wire.

Table 1. Physical properties of some reinforcement materials.\(^3,4\)

<table>
<thead>
<tr>
<th>Material</th>
<th>(\rho) (g cm(^{-3}))</th>
<th>(\sigma_f) (GPa)</th>
<th>(E_A) (GPa)</th>
<th>CTE (ppm K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Glass fiber</td>
<td>2.6</td>
<td>3.5</td>
<td>72</td>
<td>0.5</td>
</tr>
<tr>
<td>Kevlar-49 fiber</td>
<td>1.4</td>
<td>3.5</td>
<td>124-130</td>
<td>-2.7</td>
</tr>
<tr>
<td>Carbon fiber</td>
<td>1.8</td>
<td>1.5-5.5</td>
<td>150-500</td>
<td>-0.1</td>
</tr>
<tr>
<td>Steel wire</td>
<td>7.8</td>
<td>4.1</td>
<td>210</td>
<td>11.1</td>
</tr>
<tr>
<td>Carbon nanotubes</td>
<td>-</td>
<td>11-63</td>
<td>270-950</td>
<td>-</td>
</tr>
<tr>
<td>Crystalline cellulose</td>
<td>1.6</td>
<td>7.5-7.7</td>
<td>110-220</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(\rho\) = density, \(\sigma_f\) = tensile strength, \(E_A\) = axial elastic modulus, CTE = Coefficient of thermal expansion

Because of its high abundance and outstanding properties, cellulose in the form of wood or wood pulp has been commercially used in an uncountable number of applications. In the last couple of centuries, the chemical modification of cellulose has allowed the increase of its application range and introduced new functionalities. For instance, new materials have been created from regenerated cellulose or cellulose acetates. More recently, a new approach for the utilization of cellulose has been the preparation of cellulose nanoparticles.
There are two different kinds of cellulose nanoparticles, cellulose nanocrystals (CNCs) and cellulose nanofibrils. In all cellulosic sources, cellulose is synthesized by cellulose synthase complexes in the form of 3-5nm wide nanofibrils, also known as elementary fibrils or microfibrils. These nanofibrils tend to aggregate because of hydrogen bonding. In wood, the CNFs form a hierarchical structure, as shown in Figure 2. To prepare cellulose nanoparticles, cellulose fibers and nanofibril aggregates are broken down by different means (e.g. mechanical treatment or chemical modification).

CNCs are isolated by hydrolysis of the disordered regions present in the CNF aggregates. The ordered regions, more resistant to hydrolysis, remain intact. The nanocrystals are also known as cellulose whiskers, due to their characteristic rod-like shape shown in Figure 3a. The size of these CNCs depends on the structure of the terminal complex that produced cellulose, i.e. on the cellulose source. They are typically 50-500 nm long and 3-20 nm wide.

When CNF aggregates are disintegrated without removing the disordered regions CNFs are released, this process is known as defibrillation. In the particular case of the cellulose from bacteria, these organisms directly extrude cellulose nanofibers into their growing medium. Since this thesis is focused on the modification of CNFs, the following subsections will explain in more detail the different kinds of nanofibrils that have been employed, a classification considering the cellulosic source has been used.
1.2.1. CNFs from wood pulp

Because of its large availability and well established industry, the most common source material of CNFs is wood. After mechanical and chemical treatment of wood chips to remove the non-cellulosic components and to break the cellular structure of wood, individualized wood cells are obtained. These cells are known in the pulp and paper industry as fibers. A wood cell or fiber consist of a thin primary cell wall and a thick secondary cell wall that is mostly cellulose. Pulp fibers are long and flexible, with a morphology that depends on the tree species. The fibers are typically a few millimeters long and 20-40 μm wide. Since pulp prepared by chemical means has higher cellulose content, this pulp is preferred as a starting material for the preparation of CNFs. Wood pulp can be treated in different approaches to break the cellulosic fibers and obtain individualized CNFs. The first successful approach to prepare CNFs was by mechanical disintegration or defibrillation. Turbak et al.\textsuperscript{8} disintegrated wood pulp fibers into CNFs by passing several times a pulp suspension through a homogenizer at high pressure. Later on, the high energy consumption of the process was decreased by Pääkkö et al.\textsuperscript{9} and Henriksson et al.\textsuperscript{10} using an enzymatic pretreatment of the wood pulp. Another approach to prepare CNFs is by chemical pretreatment of the wood pulp followed by mechanical disintegration. The purpose of the chemical modification is to introduce charged groups at the surface of the nanofibrils that facilitate defibrillation. Using chemical methods, well individualized CNFs have been prepared by TEMPO-mediated oxidation of wood pulp followed by a mild mechanical treatment.\textsuperscript{11} Wågberg et al.\textsuperscript{12} used carboxymethylation as a pretreatment to prepare CNFs. When no chemical pretreatment is used, CNFs tend to show fibrils aggregates, which are typically 5-30 nm wide and a few micrometers long\textsuperscript{13} (Figure 3b). The chemical pretreatment generally causes a more uniform width distribution of the CNFs, with a few fibril aggregates and thin nanofibrils (3-15 nm).\textsuperscript{11, 14}

\textbf{Figure 3.} Scanning electron microscope in transmission mode images of CNCs (a), CNFs from enzymatically pretreated wood pulp (b), BC nanofibers (c), and CNFs from primary cell wall (d). Scale bar 100 nm.
1.2.2. CNFs from bacteria

Bacteria from the genus *Acetobacter* naturally secrete CNFs. Since these bacteria are aerobic, the biological function of bacterial cellulose (BC) is thought to be as a flotation aid. BC is extracellularly synthesized in a virtually pure form, without any other associated polysaccharides as occurs in cellulose from plants. Therefore, BC is often used as a model for the study of cellulose. Moreover, BC possesses high crystallinity. CNFs from bacteria have a singular morphology compared to CNFs from other sources owing to the unique cellulose biosynthetic machinery of bacteria. As shown in Figure 3c, BC nanofibers are larger than CNFs from wood or primary cell walls. With a characteristic morphology, BC nanofibers are several micrometers long and exhibit a rectangular cross-section of typically 70-150 nm wide and 7 nm high. The BC nanofibers are continuously extruded by the bacteria, creating a three-dimensional network nanostructure known as pellicle. BC pellicles possess outstanding mechanical properties and great water-holding capacity. Furthermore, they can be shaped in situ by modifying the container where the bacteria grow. When a homogeneous water suspension of BC nanofibers is desired, the BC pellicle has to be disrupted by mechanical means to liberate the nanofibers. Due to its unique properties, BC has mostly been used for biomedical applications such as blood vessels or wound dressings.

1.2.3. CNFs from primary cell wall

Wood is mainly composed of cells with a thick secondary cell wall. Secondary cell walls possess a high content of cellulose, which is embedded in a lignin-rich matrix. Other plant sources of cellulose are mainly composed of cells with only primary cell wall. Primary cell walls have different composition than secondary cell walls. The morphology and chemical properties of CNFs isolated from different primary cell wall sources (e.g. fruit tissues, potato, sugar beet, onion and quince seed mucilage) have been studied. As shown in Figure 3d, CNFs from primary cell wall are thinner than those from secondary walls. Even though they are partially crystalline, the crystallinity of CNFs from plant primary cell walls is much lower than that of CNFs from other sources such as BC or wood pulp.

1.3. Surface modification of CNFs

CNF characteristics such as colloidal stability, hydropillicity, and processability among others, will be determined by the chemical structure present at the surface of CNFs. Furthermore, the mechanical properties of the resulting material or
composite will be affected by the surface structure of the CNFs as well. This effect is even more dramatic for CNFs than in the traditional case of cellulosic fibers modification in the pulp and paper industry, due to the large specific surface area of nanofibers. The aim of surface modification on CNFs is to tailor the nanofibril properties by using the hydroxyl groups naturally present at the CNF surface to introduce other functionalities. Even though there are many different approaches to perform a surface modification on CNFs, those could be categorized in 2 main strategies: modification via in-situ synthesis and via topochemical surface modifications.

Figure 4. Examples of surface modified CNFs from wood pulp fibers: Enzymatic CNFs (1), TEMPO-oxidized CNFs (2). Xyloglucan adsorption on CNFs (1a), Silylation of CNFs (1b), Ionic adsorption of methoxypol yethylene glycol amine onto carboxylated CNFs (2a), and covalent modification of carboxylated CNFs by amidation (2b).
Topochemical reactions on CNFs can be used to tune the functionalities and properties of CNFs. “Solid-state reaction are controlled by the relatively fixed distances and orientations, determined by the crystal structure, between potentially reactive centers.” For CNFs, these reactive centers are hydroxyl groups periodically distributed at the surface of the nanofibril. In addition to hydroxyl groups, CNFs prepared using chemical pretreatments on pulp fibers, such as TEMPO-mediated oxidation or carboxymethylation, will also carry carboxyl or carboxymethyl groups, respectively. Depending on the nature of the bound formed during a topochemical reaction, these reactions can be divided into non-covalent and covalent modifications. An example of the different types of topochemical modifications that can be performed on CNFs is shown in Figure 4.

Pretreatments on wood pulp fibers: The modification of CNFs can take place before their fibrillation. The chemical pretreatment of pulp fibers leads to the loosening of the micrometric structure and to the isolation of already modified CNFs. A good example of surface modification by pretreatment is the TEMPO-mediated oxidation of wood pulp. This chemical reaction selectively introduces negatively charged carboxyl groups at the surface of the CNFs in the pulp fibers. This pretreatment facilitates enormously the individualization of CNFs, decreasing the energy consumption of the disintegration process. Because of this preparation route, the resulting CNFs exhibit the carboxylic functionality at their surface, as shown in Figure 4-2. Another example of chemical reaction on pulp fibers that facilitates fibrillation and leads to modified CNFs is carboxymethylation. In this case, the resulting CNFs present the carboxymethyl functionality at their surface. The pulp fiber can be also quaternized with 2,3-epoxypropyl trimethylammonium chloride. The resulting CNFs have cationic trimethylammonium groups at their surface.

Non-covalent modification on CNFs: In this type of modification, weak chemical bonds are created by physical interactions such as hydrogen or ionic bonds. Polyelectrolytes and polysaccharides can be adsorbed onto the surface of CNFs by Van der Waals interactions or multivalent hydrogen bonding, as in the case of carboxymethyl cellulose (CMC) and xyloglucan (Figure 4-1a). The adsorbed molecule might not be the final functionality; it can be also used as a tool for further modification. Hydroxyl groups at the surface of CNFs can act as a nucleating site for the growth of inorganic nanoparticles. Moreover, charged groups at the surface of CNFs can bind to species with opposite charged ions by ionic bonding. As shown in Figure 4-2a, positively charged polymers such as methoxypolyethylene glycol amine can be grafted to carboxylated CNFs by ionic
interaction. The same reactive sites can be used to bind cationic surfactants and cationic block-copolymers. In a similar manner, negatively charged carboxymethyl groups found at the surface of CNFs prepared by carboxymethylation of wood pulp can be used to bind positively charged polyelectrolytes.

**Covalent modifications on CNFs:** In this case, a covalent bond between the surface functional groups on CNFs and another molecule is created. A wide range of chemical modifications have been performed to introduce the desired functionality at the CNF surface, e.g. silylation (Figure 4-1b), acetylation, amination (Figure 4-2b), and polymer grafting. In this manner, the surface of CNFs can be tuned to achieve properties such as hydrophobicity and oleophobicity. Nevertheless, these types of heterogeneous reactions tend to decrease the crystallinity of the CNFs. The decrease in crystallinity might cause a reduction of the mechanical performance and reinforcing potential of CNFs.

**1.4. Modification of CNFs during biosynthesis**

The modification of CNFs can be achieved by introducing an alteration in the media where the CNFs are synthesized. This method, also known as *in situ* or *in vitro* modification, has been used for the modification of BC and plant cell suspension cultures. When cultivating *Acetobacter*, different water-soluble compounds (e.g. calcofluor, carboxymethyl cellulose, hydroxyethyl cellulose, chitosan, and poly(vinyl alcohol)) have been added to the growing medium of the bacteria. These compounds physically interact *in situ* with the surface of the CNFs when they are being extruded, causing major differences in the morphology and properties of the nanofibrils. In a similar manner, different enzymes or enzymatic domains can be added to plant cultures *in vitro*, in order to interact with the CNF formation just after its biosynthesis.

A step further than *in vitro* modifications is the genetic modification of the cellulosic source, i.e. *in vivo* modification. In this case, the cellulose source can be genetically engineered to up-regulate or down-regulate the production of certain naturally occurring polysaccharides or enzymes. Furthermore, the transgenic organism can be designed to overexpress new proteins that will interact with the cellulose surface during its biosynthesis. For example, Kawano et al. engineered an *Acetobacter xylinum* mutant that overproduced an endo-beta-1,4-glucanase. The mutant exhibited a modified ribbon structure. Thus, this enzyme was suggested to affect the assembly and crystallization of cellulose nanofibers after cellulose biosynthesis. Poplar trees were genetically modified to overproduce a carbohydrate-
binding module from family 3, thus modifying in vivo the CNFs. This modification caused structural changes in the fibers of the transgenic trees, and increased mechanical properties of the resulting paper.

1.5. CNF-based materials

Cellulose nanoparticles, with one of their dimensions in the nanoscale, can be used to build up nanostructured cellulosic materials such as hydrogels, thin films, nanopapers, and aerogels and foams. Moreover, they can be utilized as reinforcement filler in conventional petrol-based or bio-based polymers.

CNF hydrogels are prepared by concentrating a CNF suspension until the desired solid content is reached. This is often done by vacuum filtration or by centrifugation. The mechanical properties CNF hydrogels can be further improved by chemical cross-linking.

CNF nanopapers, also known as films, are prepared by evaporation of all the liquid in a CNF suspension or hydrogel. The nanopapers can be prepared by solvent casting or using a faster method mimicking paper-making. CNF nanopaper exhibits high toughness, optical transparency and thermal stability. Moreover, a preferential orientation of TEMPO-oxidized CNFs in cellulose nanopapers can be obtained by cold drawing of hydrogels prior drying, or by rewetting and stretching of nanopapers. The preferential orientation of the nanofibrils in the material will cause anisotropic properties resulting in improved tensile strength and stiffness along the preferential direction of the CNFs.

CNF foams are typically prepared by sublimation of ice crystals of a frozen CNF suspension. This process is known as freeze-drying or lyophilisation. By using different solvents the porosity of CNF foams can be tuned. More recently, foams have been prepared by drying water-air emulsions stabilized by surface-modified CNFs. Due to their high porosity, foams are lightweight materials with high impact energy absorption and excellent thermal and sound insulation. The porosity of the dry CNF materials can be further increased by using supercritical drying. In this drying technique, the solvent of the CNF suspension is first exchanged to carbon dioxide. Then, pressure and temperature are raised until a supercritical fluid state is reached. Subsequently, the pressure is released causing the evaporation of the carbon dioxide. This process leads to a highly porous material that preserves the structure of the CNF suspension before drying. This kinds of materials are known as aerogels.
1.6. Applications of CNFs

CNFs can be used as reinforcement filler for biopolymers such as natural rubber,\(^{65}\) starch,\(^{66-68}\) and polylactic acid.\(^{69}\) Moreover, CNFs can be used to reinforce commodity polymers such as polypropylene\(^{70, 71}\) and polyethylene.\(^{72}\) Nevertheless, a surface modification of the CNFs or the addition of a compatibilizer is necessary to allow good dispersion and stress transfer between the polar CNFs and the non-polar polymer matrix.\(^{30, 65}\)

Conductive and strong nanopapers can be prepared by the incorporation of carbon nanotubes onto a CNF matrix.\(^{73, 74}\) It is also possible to prepare conductive CNF aerogels by the incorporation of carbon nanotubes\(^{73}\) or polypyrrole.\(^{75}\) Foldable antennas for the fabrication of small-size electronic devices have been prepared by printing CNF nanopapers with silver nanoparticles ink.\(^{76, 77}\) All-polymer based batteries have been produced using CNFs from algae that were coated with polypyrrole.\(^{78}\) Furthermore, CNF aerogels can be used as precursors for the preparation of carbon aerogels.\(^{79}\) In this manner supercapacitors\(^{80}\) and supports for Li-ion battery anode materials\(^{81}\) can be prepared. In addition, magnetic CNF aerogels and nanopapers can be prepared by decorating the nanofibrils with magnetic nanoparticles.\(^{29, 82}\) This new materials have been used in the construction of loudspeakers or electronic actuators.

Cellulose nanopapers are ideal substrates for flexible displays\(^{83}\) and thin-film transistors arrays, because of their optical transparency and thermal stability.\(^{84, 85}\) The range of optical applications for CNF nanopapers might be broaden by the possibility to tune the diffuse light scattering of the films\(^{85, 86}\) since a large haze (i.e. ratio between diffuse transmission and total transmission) is beneficial for solar cells design and anti-glare outdoors displays, while low haze is required for indoor displays.

High gas and oil barrier properties are intrinsic properties of CNF films.\(^{87, 88}\) The barrier properties of CNF materials can be further increased by the addition of other nanoparticles into a CNF matrix. For instance, CNF nanocomposites with clay nonoplatelets have been shown to possess very interesting mechanical and gas barrier properties, as well as fire retardancy.\(^{89-91}\) Talc platelets in a CNF matrix have been found to further improve the barrier properties of CNF films.\(^{92}\)

CNF foams and aerogels are good insulators owing to their high porosity, thus they can be used in applications such as thermal and acoustic insulation.\(^{93}\)

The charge present at the surface of some types of CNFs makes them a promising material for the removal of ionic substances from water. Due to the negative charge present at the surface of carboxylated CNFs, this nanofibrils have been used for the
removal of cationic dyes\textsuperscript{93} or radioactive ions\textsuperscript{94} from water. Moreover, the functionalization of CNFs towards hydrophobic materials allows the extraction of organic pollutants and oils from contaminated water. This can be achieved by silylation\textsuperscript{34,95,96}, or by carbonization of CNF aerogels.\textsuperscript{97}

CNFs are of great interest in the preparation of materials for biomedical applications, owing to their biocompatibility. BC natural hydrogels can be used as blood vessels,\textsuperscript{17} or wounds and burns dressing.\textsuperscript{16,18} The biocompatibility and antibacterial activity of cellulose can be improved by the addition of chitosan onto a CNF matrix.\textsuperscript{41,98} Bifunctional recombinant proteins with a cellulose binding-domain and an adhesive peptide domain were adsorbed onto BC with a resulting improvement in cell adhesion.\textsuperscript{99} Furthermore, hydroxyapatite crystals can be grown into CNF substrates for biomedical applications.\textsuperscript{100,101} In addition, the introduction of silver nanoparticles onto CNFs has shown to produce materials with antibacterial activity.\textsuperscript{102,103}

\section*{1.7. Specific aims of the current study}

This thesis consists of five publications with focus on the following four specific aims: optimizing the conditions for surface modification of CNFs, introducing new functionalities to CNFs, understanding the structure-property relationship between CNFs and CNF-based materials, and exploring new applications for modified CNFs.

The optimization of the reaction conditions is vital for an efficient and homogeneous surface modification of CNFs. Therefore, in Paper I, the adsorption of carboxymethyl cellulose (CMC) onto CNFs was carried out at both room and high temperature. In Paper III, the grafting of polyethylene glycol (PEG) onto CNFs was also performed at two different temperatures. The effect of the temperature on the yield of the modification was studied. In Paper II, the influence of the NaOH concentration on the surface quaternization of CNFs was investigated. Further, to study the feasibility of performing the modification of CNFs prior to their defibrillation, the covalent modification reactions with trimethylammonium chloride or PEG molecules were directly performed onto wood pulp fibers.

To introduce new functionalities to CNFs, negatively charged polymer (CMC), permanent cationic groups (trimethylammonium), positively charged deacetylated chitin nanocrystals (ChNCs, in Paper IV), and pendant uncharged polymer (PEG)
have been either adsorbed or covalently linked to the surface of CNFs. The influence of these moieties on the final properties of CNFs and CNF-based materials has been studied.

The structure of nanomaterials has a direct impact on their properties. The relationship between the structure of CNF-based materials and their properties was investigated. The effect of the structure of BC/ChNC nanopapers on their mechanical and antibacterial properties was characterized. The effect of PEG-grafting onto CNFs on the alignment of CNFs and the mechanical and optical properties of the resulting material was also studied. In addition, the effect of the genetic engineering of tobacco cells on the structure of CNFs and on the mechanical properties of nanopapers from primary cell wall CNFs was investigated (Paper V).

Finally, it was also a goal of this thesis to propose new applications for CNFs. To do so, the water-redispersibility of dry CNFs adsorbed with CMC, the water absorbency and dye removal capacity of quaternized CNFs, the light diffraction of CNF-g-PEG ribbons, the antibacterial activity of BC/ChNC nanopapers, and the mechanical properties of CNFs from genetically modified tobacco cells were studied.
2. EXPERIMENTAL

2.1. Materials

Commercial never-dried softwood sulphite pulp (86% cellulose, 14% hemicellulose, <1% lignin, DP 1200) was provided by Nordic Paper (Sweden). Tobacco BY-2 cell suspension cultures (Nicotiana tabacum L. cv. Bright Yellow, DSMZ PC-1181) expressing a family 3 cellulose-binding module (CBM3) from Clostridium thermocellum cellulosome integrating protein (GenBank accession number ABN54273) had been prepared according to Leijon et al.\textsuperscript{104}. Carboxymethyl cellulose (CMC, sodium salt, $M_w=2.5 \times 10^5$, DS=0.90), 2,2,6,6,-teramethyl-1-piperidinyloxy radical (TEMPO), methoxy polyethylene glycol amine (PEG-NH$_2$, $M_w=750$), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), $\alpha$-chitin powder from shrimp shells, and all other chemicals and materials used in this work were purchased from Sigma-Aldrich, VWR, or Ted Pella and used without further purification.

2.2. Preparation of CNFs adsorbed with CMC (Paper I)

Enzymatically pretreated CNFs prepared from softwood sulphite pulp were obtained following a protocol adapted from a previously reported method by Henriksson et al.\textsuperscript{10}. The wood pulp was beaten to open the fiber structure and resuspended in phosphate buffer at pH 7. Then, an endoglucanase preparation (FiberCare R, Novozymes, Denmark) was added to the suspension in the optimal ratio specified by the manufacturer, and the suspension was incubated at 50 °C. After 2 hours, the reaction was stopped by washing the suspension thoroughly with deionized water by filtration. The remaining enzyme was denaturized by adding boiling water to the filtrate and incubating at 90 °C for 30 min. Finally, the enzymatically pretreated pulp suspension was homogenized with 8 passes through a microfluidizer (M-110EH, Microfluidics Ind., USA), a 2 wt% water suspension of individualized CNFs was obtained.

Enzymatic CNFs were irreversibly adsorbed with CMC in water suspension at two different temperatures: room temperature (RT), and high temperature (HT). In brief, different amounts of CMC were added to a CNF suspension, with a final CNF content of 1 wt%. The resulting suspensions were then homogenized with an Ultra-Turrax. For the adsorption at RT, the suspensions were stored at ambient
conditions overnight prior to thoroughly washing by centrifugation to remove the unbound CMC. When the adsorption was carried out at HT, the suspensions were autoclaved at 121 °C for 25 minutes, cooled down at room temperature overnight, and washed by centrifugation. The samples were coded as CNF-CMC-RT-x or CNF-CMC-HT-x, where x is the initial amount of CMC added to the CNF suspension in mg CMC/g CNFs.

2.3. Preparation of surface quaternized CNFs (Paper II)

As summarized in Figure 5, surface quaternized CNFs were prepared by quaternization of pulp fibers followed by mechanical disintegration. The alkali-activated hydroxyl groups present at the surface of the fibers were covalently modified by nucleophilic addition of the epoxy moiety in glycidyltrimethylammonium chloride. Briefly, never-dried commercial softwood sulphite pulp was subjected to a mechanical beating treatment to open the fiber structure. Then, the pretreated pulp was resuspended in a NaOH solution, with a final concentration of 5 wt% and 7.5 wt% for the NaOH and the pulp, respectively. A known amount of glycidyltrimethylammonium chloride was added to the suspension and the reaction was performed at 65 °C for 8 hours. The reaction was stopped by neutralizing the suspension with HCl, and the quaternized pulp fibers were washed thoroughly with deionized water by filtration. The quaternized pulp fibers were resuspended in water and homogenized with 1 pass through a microfluidizer (M-110EH, Microfluidics Ind., Newton, MA) to obtain a suspension of fully individualized quaternized CNFs with trimethylammonium chloride groups at their surface.

![Figure 5](image-url)  
**Figure 5.** Schematic illustration of the preparation of quaternized CNFs from pulp fibers.
2.4. Preparation of CNFs grafted with PEG (CNF-g-PEG) (Paper III)

The preparation route for CNF-g-PEG is illustrated in Figure 6. In the first step, wood pulp fibers were carboxylated by TEMPO-mediated oxidation following a method by Saito et al. \textsuperscript{105} Briefly, TEMPO (16 mg/g cellulose) and NaBr (100 mg/g cellulose) were added to a water suspension of pulp fibers (1 wt%) under vigorous stirring at room temperature. NaClO (7.5 mmol/g cellulose) was added to the suspension dropwise while maintaining a constant pH of 10 using 0.1 M NaOH. When the pH of the suspension became constant, the oxidized pulp fibers were thoroughly washed with deionized water by filtration.

The never dried oxidized fibers were then resuspended in water to a solid content of 1.5 wt%. The modified pulp fibers had a carboxylate content of 1.6 mmol g\textsuperscript{-1}, as measured by conductimetric titration. The carboxylated pulp fibers were further modified with amino-terminated PEG (PEG-NH\textsubscript{2}) by carbodiimide-mediated amidation with a protocol adapted from a previously reported method.\textsuperscript{106, 107} EDC (614 mg, 3.2 mmol) and NHS (276 mg, 2.4 mmol) were dissolved in 1.5 wt% water suspension of carboxylated pulp fibers (1 g pulp). Solid PEG-NH\textsubscript{2} (2.4 g) was added to the mixture and stirred until complete dissolution. The suspension was then stirred either at 22 °C or 37 °C for 24 hours. During the reaction, a slightly basic pH (7.5-8) was maintained by adding small amounts of 0.1 M NaOH or HCl. The reaction was stopped by decreasing the pH to 1 and the suspension was purified by dialysis against deionized water.

The resulting PEG-grafted pulp fibers were resuspended in water to a concentration of 0.2 wt% and disintegrated using a high speed kitchen blender (Vita-Prep 3 model, Vita-Mix Corp., USA) for 5 minutes, followed by a ultrasonication treatment using a Branson S-250A (USA) sonicator at 70% output control for 2 minutes. A transparent suspension of CNF-g-PEG was obtained.
2.5. Preparation of BC modified with ChNCs (Paper IV)

2.5.1. BC preparation

Hestrin-Scheramm (HS) liquid medium\textsuperscript{108} was inoculated with pre-cultured \textit{Acetobacter aceti} (strain AJ-12368) bacterial cells. The bacteria were cultivated at 27 °C for 14 days to produce a thick BC pellicle. To remove the bacteria and the medium that remained in the pellicle, a washing with 1 wt% NaOH at 80 °C was performed 3 times. Then, the pellicle was immersed in running deionized water for 3 days. Thus, a three-dimensional network of pure CNFs was obtained. To prepare a water suspension of BC nanofibers, the pellicle was disrupted using a high speed kitchen blender (Vita-Prep 3 model, Vita-Mix Corp., USA).

2.5.2. ChNC preparation

By acid hydrolysis: Chitin from shrimp shells was hydrolyzed with HCl to prepare ChNCs following a protocol adapted from Revol et al.\textsuperscript{109}. In summary, 5 g of chitin powder was added to 100 ml of 3M HCl solution at 105 °C. After 2 hours, the reaction was stopped by diluting the suspension with 2 L deionized water. The
sedimented chitin was washed with deionized water by centrifugation until pH 2, when a turbid supernatant appeared. This supernatant was dialyzed against deionized water until neutral pH was reached. Finally, the suspension was sonicated for 1 minute, obtaining a stable suspension of ChNCs (A-ChNCs).

**By TEMPO-mediated oxidation:** ChNCs were prepared by TEMPO-mediated oxidation using a protocol from Fan et al.\textsuperscript{110} with a few modifications. Briefly, 10 g of chitin powder from shrimp shells was added to 1L of water solution containing 0.16 g of TEMPO and 1 g of NaBr. A 12 wt% solution of NaClO (46.53 g) was added to the suspension dropwise. When necessary, 0.5 M NaOH was added to maintain a constant pH of 10. When the pH of the suspension became stable, the suspension was washed thoroughly with deionized water by filtration. The insoluble fraction was collected, resuspended in deionized water and homogenized with 5 passes through the microfluidizer. A transparent and stable suspension of TEMPO-oxidized ChNCs (T-ChNCs) was obtained.

**By partial deacetylation:** Partially deacetylated ChNCs (D-ChNCs) were prepared from shrimp shell chitin powder using a procedure adapted from Fan et al.\textsuperscript{111}. 10 g of chitin powder was added to 100 mL of 33 wt% NaOH solution. The suspension was heated at 95 °C for 3 hours under vigorous stirring. The reaction was stopped by dilution with 2 L of deionized water. The partially deacetylated chitin that precipitated was separated by decantation and washed with deionized water by filtration until the filtrate reached neutral pH. The residue was resuspended in deionized water and blended with a high speed kitchen blender for 10 minutes. The suspension was centrifuged at 3200 \(g\) for 10 minutes, obtaining a stable suspension of D-ChNCs as supernatant.

### 2.5.3. BC modification with D-ChNCs

D-ChNCs were incorporated into BC networks by two different routes: \textit{in situ} biosynthesis (BC/D-ChNC-i) and post-modification (BC/D-ChNC-p).

**In situ modification:** D-ChNCs were added to 30 mL of H-S media. After inoculation with pre-cultured bacterial cells (5 mL), the final concentration of D-ChNCs in the suspension was 0.2 wt%. A thick BC/D-ChNC pellicle was produced after the incubation of the cultures at 27 °C for 2 weeks. To purify the nanocomposite, medium and bacteria remaining in the pellicle, as well as unbound D-ChNCs, were removed by washing 4 times in 1 wt% SDS solution at 80 °C, followed by the immersion of the pellicle in running deionized water for 3 days.
Post-modification: In this route, a water suspension of BC nanofibers (0.42 wt%) was mixed with a suspension of D-ChNCs to prepare 150 g suspension with a solid content of 0.2 wt%. The amount of D-ChNCs in the suspension was adjusted to obtain a final concentration of 0, 10, 20, 50 or 100 wt% of D-ChNCs in the dry nanocomposite. Then the suspension was filtered and dried to prepare a nanopaper (see section 2.7.2).

2.6. Preparation of CNFs from CBM3-transformed tobacco cells (Paper V)

Wild-type and genetically modified tobacco cells overexpressing CBM3 were kindly supplied by Felicia Leijon. The cellulose from the suspension-cultured tobacco cells was extracted using a protocol adapted from Wilson et al.\textsuperscript{112}. Briefly, 200 g of washed cells were resuspended in deionized water (to a total of 500 g). The suspension was placed in an oven at 65 °C. 4 g of NaClO\textsubscript{2} and 1.375 ml of acetic acid were added to the suspension every 15 min, for a total of 4 additions. The suspension was removed from the oven and incubated at room temperature overnight. Subsequently, the suspension was washed 4 times with deionized water using an Avanti J-26XP centrifuge (Beckman Coulter Corp., USA) at 17700 g for 15 min. The sample was resuspended in deionized water (to a total of 300 g). After the addition of 30 mL of 100 mM NaAc at pH 5, the suspension was incubated at 100 °C for 1 hour. Then, 3 ml of 2M K\textsubscript{2}CO\textsubscript{3}/0.3M CDTA was added to the suspension prior to further incubation at room temperature overnight. The sample was successively washed twice by centrifugation as above, resuspended in 5.6 wt% KOH (to a total of 300 g) and incubated overnight at 4 °C. Finally, the sample was washed with deionized water by centrifugation until neutral pH was reached. The resulting suspension of extracted cellulose had a solid content around 0.2 wt%. The remaining cell structure of the cellulose particles in suspension was disrupted using a sonicator (Sonifier 250, Branson Ultrasonics Corp., USA) for 3 or 10 min. A stable suspension of individualized CNFs from primary cell wall was obtained.

2.7. Preparation of CNCs from CBM3-transformed tobacco cells (Paper V)

Freeze-dried cellulose extracted wild-type and CBM3-transformed tobacco cells (100 mg) was hydrolyzed in 1 ml of 64 wt% H\textsubscript{2}SO\textsubscript{4}. The reaction was carried out at 45 °C under stirring. After 45 minutes, the reaction was stopped by dilution with
deionized water. The resulting suspension was dialyzed against deionized water using regenerated cellulose dialysis membranes (Spectrum Spectra/Por, 12000-14000 molecular cut-off) for 7 days until constant neutral pH was achieved. Mixed bed ion-exchange resin (Dowex Marathon MR-3 hydrogen and hydroxide form) was added to the cellulose suspension for 48 h and then removed by filtration. Finally, the suspension was sonicated for 1 minute (with cooling in an ice bath) to create CNCs colloidal suspension.

2.8. Preparation of CNF-based materials

2.8.1. Drying and redispersion of CNFs adsorbed with CMC (Paper I)

Water suspensions of CNFs and CNFs adsorbed with CMC (CNF-CMC) were dried in an oven at 80 °C until constant weight was reached. Typically, the dry samples were resuspended in deionized water to a solid content of 1 wt% and stirred overnight at ambient conditions. Subsequently, the suspensions were homogenized using an Ultra-Turrax mixer for 15 minutes.

2.8.2. Preparation of nanopapers (Papers I-V)

Nanopapers were prepared by drying free-standing hydrogels prepared from CNF suspensions. Typically, a CNF water suspension was degassed and vacuum filtered on a glass filter funnel (Ø 7.2 cm) using a filter membrane (DURA-PORE®, 0.22 or 0.65 μm, DVPP, Millipore, Ireland) until a free-standing hydrogel was formed. The hydrogel was placed between woven metal cloth and then between two paper carrier boards, and dried using an automatic sheet former (Rapid Köthen, RK3A-KWT PTI, Germany) for 20 minutes at 93 °C and at a pressure of about 70 mbar.

Nanopapers of in situ modified BC/D-ChNC pellicles (Paper IV) were prepared by air-drying the pellicles at ambient conditions.

2.8.3. Preparation of oriented ribbons (Paper III)

Oriented ribbons of TEMPO oxidized CNFs and CNF-g-PEG samples were prepared from free-standing hydrogels with solid contents of 80 to 85%, that had been prepared as explained in the previous section. The hydrogels were cut into 0.5 cm wide strips. These strips were stretched using a universal testing machine (Instron 5944, USA) following the same procedure as the previously reported by
Sehaqui et al.,\textsuperscript{60} with minor modifications. The strips were stretched 40\%, from 5 to 7 cm, at a crosshead speed of 1 mm min\textsuperscript{-1}. Subsequently, the samples were quickly dried using the same process as for the drying of nanopapers.

2.9. Characterization techniques

2.9.1. Viscosity (Paper I)

The viscosity of cellulosic suspensions was studied with a cone/plate rheometer (RVDV-III, Brookfield). Viscosity as a function of the shear rate was measured using a cone CP-40 with an angle of 0.8°, at 25 °C.

2.9.2. Conductimetric titration (Papers I-IV)

The carboxylate groups present in T-ChNCs (Paper IV), CMC adsorbed on CNFs (Paper I), and TEMPO-oxidized CNFs (Paper III) were measured by conductimetric titration. Typically, 0.1g of sample was diluted to a 0.1 wt\% with Milli-Q water. The pH of the suspension was adjusted to 2.5-3 with 0.1 M HCl using a pH station (FiveEasy, Mettler-Toledo), and monitored during all the titration. The conductivity of the sample was followed using a conductimetric station (SevenCompact, Mettler-Toledo) while titrating with 0.01 M NaOH until the suspension reached pH 11. The carboxylate content was calculated from the titration curve according to previous studies.\textsuperscript{107, 113}

The amount of trimethylammonium chloride groups present in quaternized CNFs (Paper II) was measured by conductimetric titration of chloride ions.\textsuperscript{114} Briefly, 100 mg of quaternized CNFs was dispersed in 100 ml of Milli-Q water and titrated with 8 mM AgNO\textsubscript{3} water solution. The titrant was added in aliquots of 0.2 mL every 60 seconds and the conductivity of the suspension was measured with a conductimetric station (SevenCompact, Mettler-Toledo). One chloride counterion was considered equivalent to the presence of one trimethylammonium group.

2.9.3. Electron Microscopy: FE-SEM, STEM, and TEM (Papers I-V)

The morphology of CNF suspensions (Papers I and V), nanopapers (Papers II and IV), and freeze-dried BC pellicles (Paper IV), was studied using a field-emission scanning electron microscope (FE-SEM) (Hitachi S-4800, Japan). For the visualization of CNFs in suspensions (Paper I and V), the suspensions were freeze-dried or dried under vacuum on the surface of carbon tape on a metal stub. For
nanopapers and dry CNF-based materials (Paper II and IV), the samples were directly fixed to a metal stub with carbon tape. When cross-sections of hydrogels were studied (Paper II), the hydrogels were freeze-fractured in liquid nitrogen, vacuum dried, and mounted in a metal split specimen holder prior to observation. All samples were coated with a thin layer of platinum-palladium with a sputter coater (Cressington 208HR) and observed at low acceleration voltage and short working distance.

The transmission detector of the scanning electron microscope (STEM) was used to observe the size and the state of aggregation of CNFs (Paper I). Briefly, a droplet of very dilute sample suspension (0.005 wt%) was deposited on a carbon coated cooper grid and stained with 2 wt% uranyl acetate. After drying at ambient conditions, the sample was visualized using a Hitachi S-4800 electron microscope in transmission mode.

Transmission electron microscope (TEM) was employed to study the morphology of CNF-g-PEG (Paper III), and CNFs and CNCs from tobacco primary cell walls (Paper IV). Specimens were prepared by depositing a droplet of dilute suspension onto a carbon-coated grid. The suspension was negatively stained with 1 wt% uranyl acetate and dried at ambient conditions. The samples were observed using a HT7700 transmission electron microscope (Hitachi, Japan) operated in high-resolution mode at 100 kV.

### 2.9.4. Tensile test (Papers I-V)

The mechanical characterization of CNF and CNF-based nanopapers (Paper I-V) was performed employing a universal testing machine Instron-5944 (Instron, USA). The samples were cut in rectangular specimens and tested at uniaxial tensile stress using a crosshead speed of a tenth of the gauge length per minute. All samples were tested at 23°C and 50% relative humidity. Elastic modulus was calculated from the slope of the stress-strain curve at low strain, while tensile strength was estimated as the stress at specimen brakeage. At least 5 specimens for each sample were measured.

### 2.9.5. Thermogravimetric analysis (TGA) (Paper II)

Thermogravimetric measurements were performed using an Exstar 6000 (Seiko Instruments Inc., Japan). All the samples were analyzed under nitrogen atmosphere in a temperature range from 30 to 600 °C. A heating rate of 10 °C min⁻¹ and a sample weight of 10 mg were used for all measurements.
2.9.6. Porosity (Paper II)

The porosity of CNF nanopapers was calculated using Equation 1, from density measurements performed with an Archimedes scale. The density of cellulose was considered to be 1460 kg m\(^{-3}\).

\[
\text{Porosity} = 1 - \frac{\rho_{\text{nanopaper}}}{\rho_{\text{cellulose}}}
\]  

Equation 1

2.9.7. Water absorption capacity (Paper II)

The capability of nanopapers prepared with surface quaternized CNFs to adsorb water was determined by weight difference after immersion in deionized water for 7 days. The following equation was employed:

\[
Q = \frac{m_2 - m_1}{m_1}
\]  

Equation 2

Where \(Q\) is the water absorbency (g g\(^{-1}\)), and \(m_1\) and \(m_2\) the weights of the nanopaper before and after immersion, respectively, in grams.

2.9.8. Adsorption of anionic dyes (Paper II)

Freeze-dried quaternized CNFs with different trimethylammonium chloride contents were added to 2.5 mg mL\(^{-1}\) solutions of congo red (C.I. 22120, Sigma-Aldrich C6767) or acid green 25 (C.I. 61570, Sigma-Aldrich 214566). After 1 minute, the nanofibrils were precipitated by centrifugation and the supernatant was analyzed with a UV-visible spectrophotometer (CARY 50 Bio, Varian). The adsorbed amount of dye (g dye/ g CNFs) was calculated from the difference in adsorption between the supernatant and the initial dye solution. Absorbance was measured at 498 nm for congo red, while the absorbance at 608 and 642 nm was measured for acid green 25. The correspondence between absorbance and concentration was calculated by fitting with a standard curve obtained with solutions of known concentration. For time-dependent studies of the dye adsorption capacity of quaternized CNF nanopapers, 30 mg of quaternized CNF nanopaper with a trimethylammonium chloride content of 1.32 mmol g\(^{-1}\) was added to 35 ml of 0.5 mg mL\(^{-1}\) congo red solution. The absorbance spectra of the solution was measured from 300 to 800 nm after different time intervals from 0 to 72 hours.
2.9.9. Optical microscopy (Paper II and Paper III)

Microphotographs in Papers II and III were captured using a Leitz Ortholux POL BK II optical microscope equipped with a Leica DC 300 CCD camera.

2.9.10. Atomic force microscopy (AFM) (Papers II-V)

Atomic force microscopy (AFM) was used to analyze the particle size of CNFs (Paper II and Paper V) and ChNCs (Paper IV). Moreover, this technique was used to observe the surface structure of CNF nanopapers (Paper V) and CNF-g-PEG ribbons (Paper III). Nanocrystal and nanofibril suspensions were diluted and spin coated on freshly cleaved mica substrates attach to magnetic stubs. After drying at ambient conditions the samples were scanned using RTESP silica cantilevers on a Nanoscope IIIa microscope (Veeco, Santa Barbara, USA) in tapping mode. Nanopapers and oriented ribbons were directly attached to magnetic stubs using adhesive tape. The samples were then scanned using the ScanAsyst method (Bruker, USA) in the Nanoscope IIIa microscope, with a ScanAsyst-air cantilever.

2.9.11. Infrared spectroscopy (Papers II-IV)

Fourier-transformed infrared spectroscopy (FT-IR) was used to study the trimethylammonium chloride content of quaternized CNFs (Paper II), the grafting of PEG onto TEMPO-oxidized CNFs (Paper III), and the incorporation of D-ChNCs onto BC (Paper IV). Typically, the dry sample was place on a MKII Golden Gate single reflection attenuated total reflectance system, and analyzed with a Perkin-Elmer Spectrum 2000 FTIR (Specac Ltd., UK).

2.9.12. Crystallinity (Papers II-IV)

Diffractograms of cellulosic (Paper II and III) and chitin samples (Paper IV) were recorded using a Philips X’Pert Pro diffractometer (model PW 3040/60). All measurements were performed in reflection mode for an angular range from 5 to 30° by steps of 0.05°. The X-ray beam ($\lambda = \text{1.5418 Å}$) was generated from a Cu Kα source at 45 kV and 40 mA. The beam was monochromatized using a 20 Mm Ni filter. Diffractograms were recorded from rotating specimens at room temperature. Typically, diffractograms were curve-fitted using a pseudo-Voigt function, and the crystallite size at different crystallographic planes was calculated using Scherrer’s equation:

\[
\text{Crystallite size} = \frac{0.9\times\lambda}{FHMW\times\cos\theta}
\]

Equation 3
Where $\lambda$ is the X-ray wavelength, FHMW is the full width at half maximum of the peak, and $\theta$ is the corresponding Bragg angle in radians.

The crystallinity index (CI) was calculated from the ratio between the area of the crystalline peak and the total reflection area.

Wide-angle X-ray diffraction (WAXD) measurements on CNF-g-PEG ribbon samples (Paper III) were performed using a Bruker Kappa-APEXII diffractometer. A Mo-K$_\alpha$ X-ray source was employed at 50 kV and 30 mA, and monochromated to $\lambda=0.71073$ Å. Two-dimensional diffraction patterns were obtained by mounting the sample either parallel or perpendicular to the incident beam, with a sample to detector distance of 60 mm. The diffractograms were analyzed using XRD$_2$DScan software.


The density of nanopapers was calculated by dividing the weight of a rectangular sample specimen by its volume as measured using a digital caliper.

2.9.14. **Elemental analysis (Paper IV)**

The composition of BC/D-ChNC-i nanocomposites was elucidated by elemental analysis using a Flash EA 1112 Series analyzer (Thermo Finnigan LLC, USA).

2.9.15. **$^{13}$C- Nuclear magnetic resonance (Paper IV)**

Cross-polarization/magic angle spinning $^{13}$C-nuclear magnetic resonance ($^{13}$C-NMR) spectra of ChNCs were recorded with a Bruker Avance AQS 300 WB instrument, operating at 7.04 T and 290 °K, with a MAS rate of 5 kHz. For all measurements, a 7 mm double air bearing probe was used.

2.9.16. **Antibacterial activity (Paper IV)**

The antibacterial activity of A-ChNCs, T-ChNCs, and D-ChNCs was tested on *Escherichia coli* XL1- Blue (*E. coli*) (Stratagene, Santa Clara, USA). Briefly, 1 mL of 0.5 wt% water suspension of ChNCs was added to 2 mL of Luria Broth (LB) medium that contained *E. coli* (address to the full paper for further details on *E.coli* suspension preparation). The final content of bacteria in suspension was $10^4$ colony-forming units (cfu). After incubation at 37 °C with agitation for 3 hours, the suspension was diluted 100 times and spread in a LB agar plate. The plate was
incubated overnight at 37 °C, and the colonies that had grown were counted manually.

The bactericidal activity of BC/D-ChNC nanocomposites was tested against \textit{E. coli} by immersing 7 mg of the nanocomposite into 3 mL of \textit{E.coli} water suspension that contained $10^5$ cfu mL$^{-1}$. The suspension was incubated at 37 °C with agitation. After 20 min, 1 hour, and 3 hours aliquots of the suspension were diluted and spread on LB agar plates. After overnight incubation at 37 °C, the colonies that had grown on the plates were counted manually.

\textbf{2.9.17. Light transmittance (Paper III and V)}

To study the turbidity of CNF suspensions in Paper V, the transmittance of 0.1 wt\% suspensions was measured in a wavelength range from 200 to 700 nm using a UV-visible spectrometer (CARY50 Bio, Varian Inc., USA). Using the same spectrometer, the light transmittance of ribbon samples in Paper III was measured in a wavelength range of 400 to 1000 nm.
3. RESULTS AND DISCUSSION

3.1. Modification of CNFs by adsorption of CMC (Paper I)

When cellulosic surfaces become in contact during drying, the strong hydrogen bonding that originates between the surfaces in absence of water causes irreversible agglomeration. This phenomenon is known as hornification or co-crystallization, and occurs during the drying of all kinds of cellulosic particles, from pulp fibers to CNCs. Because of the inconvenience of storing or transporting large volumes of water together with the cellulose nanoparticles, several methods to obtain water-redispersible dry nanoparticles have been developed. An indirect method to avoid hydrogen bonding between CNFs during drying is the addition of sodium chloride to the CNF suspension before freeze-drying. A more direct method is the surface chemical modification of CNFs. For example, Eyholzer et al. covalently modified the surface of CNFs by carboxymethylation to produce water-redispersible nanofibrils. Nevertheless, non-covalent surface modifications such as adsorption are less harsh towards the crystalline structure of the CNFs. Adsorption of CMC onto pulp fibers was extensively studied by Laine et al. and is of great interest for various paper products. CMC is also used as redispersing aid in commercially available redispersible microcrystalline cellulose. Lowys et al. reported that water-redispersed CNF composites containing 30% of CMC formed stable suspensions with rheological properties equal to those of never dried CNFs. In Paper I, the adsorption isotherm of CMC onto CNFs in water suspension at two different temperatures was studied. The effect of the CMC adsorption on the redispersibility of dry CNF-CMC was investigated.

3.1.1. Adsorption of CMC onto CNFs

The adsorption isotherm of CMC onto enzymatic CNFs was obtained at two different temperatures: room temperature (RT, 22 °C) and high temperature (HT, 121 °C). As shown in Figure 7, the adsorption of CMC onto CNFs at RT showed a unfavorable behavior, following a type III isotherm according to the classification of Brunauer et al. This behavior was expected, since the anionic CMC is adsorbing to slightly negatively charged CNFs. Thus, the irreversible adsorption of CMC onto CNFs relies on the attachment of unsubstituted regions along the CMC chain by hydrogen bonding, in a similar manner as in the adsorption on pulp fibers of non-ionic polymers such as methylcellulose. Surprisingly, the adsorption
at HT was more efficient, and showed a type II or BET isotherm, characteristic of a multilayer adsorption on a non-porous material.\textsuperscript{121, 122} For low initial amounts of CMC added (5-20 mg CMC/g CNFs) at HT, the adsorption of CMC onto CNFs was almost complete, with 17 mg of CMC adsorbed for the sample CNF-CMC-HT-20. The adsorption efficiency for intermediate amounts of added CMC (50 to 200 mg of CMC) drastically decreased, suggesting the saturation of the CNF surfaces with negative charges from the adsorbed CMC. For high amounts of CMC added (300 and 400 mg) the adsorption of CMC onto the CNFs increases again, causing the typical shape of a BET isotherm. Taking as value for a saturated surface an adsorption of 23 mg CMC/g CNFs (CNF-CMC-HT-100), to achieve a similar amount of CMC adsorbed at RT an initial addition of three times as much CMC compared to HT is needed (i.e. CNF-CMC-RT-300).

Considering an average diameter of 10 nm for the enzymatic CNFs, the adsorption of 23 to 59 mg CMC/g CNFs (CNF-CMC-HT-100 and CNF-CMC-HT-400, respectively), corresponds to a surface coverage in the range of 0.09 to 0.22 mg CMC/m\textsuperscript{2}. This surface coverage is similar to that previously reported for the adsorption of CMC onto regenerated cellulose films studied using surface plasmon resonance by Liu et al.\textsuperscript{123} (0.33-0.34 mg CMC/m\textsuperscript{2}). The adsorbed amount of CMC onto CNFs in water suspension could be possibly further increased by the addition of cations to the suspension.\textsuperscript{117, 123, 124}

![Figure 7](image.png)

**Figure 7.** Amount of CMC adsorbed onto CNFs as a function of the added amount of CMC in water suspensions of CNFs at HT and RT.
3.1.2. Water-redispersible dry CNF-CMC

CNF-CMC samples were dried in an oven at 80 °C to constant weight. The resulting dry composite (Figure 8A) was resuspended in water and redispersed by a mild homogenization treatment. Samples with high amounts of CMC adsorbed could be readily redispersed to high concentration suspensions, with a solid content up to 5 wt% (Figure 8B, redispersed CNF-CMC-HT-100). This procedure is easier than the usual methods to prepare suspensions with high CNF content, such as filtration\(^9\) or centrifugation.\(^{62}\) After water redispersion of dry CNF-CMC samples, the capacity of these to form a stable suspension as a function of the adsorbed CMC was studied by a sedimentation test. As shown in Figure 8C, the stability of redispersed CNF-CMC suspensions increased with increasing the adsorbed amount of CMC. Samples with low amounts of CMC adsorbed (CNF-CMC-HT-20 (c), CNF-CMC-HT-50 (d), CNF-CMC-RT-100 (f), and CNF-CMC-RT-200 (g)) precipitated before 24 hours. Only redispersed samples with 23 mg CMC/g CNF or higher CMC contents (CNF-CMC-HT-100 (e), and CNF-CMC-RT-300 (h)) remained stable after one day at rest. As expected, the threshold of adsorbed CMC necessary for complete redispersibility (i.e. 23 mg CMC/ g CNFs, or 2.3 wt\%) agrees with the amount of CMC adsorbed to form a saturated layer on the CNF surface. Thus, a surface saturated with negative charge is necessary to avoid hydrogen bonding between CNFs during drying. The amount of CMC necessary for complete redispersibility, 23 mg CMC/g CNFs, corresponds to a total surface carboxyl content of 0.185 mmol/g CNFs, i.e. a degree of substitution (DS) of 0.03. This value is lower than the DS of water-redispersible CNFs prepared by carboxymethylation (DS 0.08-0.22),\(^{116}\) suggesting that a non-covalent modification of the CNFs is more homogeneous than the covalent modification by carboxymethylation.
The morphology of the particles in suspension after the redispersion of dried CNF-CMC samples was studied by FE-SEM. Figure 9 shows the images captured after vacuum drying CNF and CNF-CMC water suspensions. The never dried CNFs sample (Figure 9a) showed a web-like open structure of well individualized CNFs. In contrast, the redispersed CNF sample (Figure 9b) showed large particles (more than 25 μm) of CNF aggregates. The aggregation of CNFs decreased with increasing the adsorbed amount of CMC. While redispersed CNF-CMC-HT-10 (Figure 9c) showed still a network of large aggregates, CNF-CMC-HT-20 (Figure 9d) exhibited already a web-like open structure of individualized CNFs with a few small aggregates. Water-redispersed CNF-CMC-HT-100 (Figure 9e), with the surface of the nanofibrils saturated by the negative charge of the adsorbed CMC, showed a web-like open structure of individualized CNFs, very similar to that of never dried CNFs.
Figure 9. FE-SEM images of vacuum dried water suspensions of never dried CNFs (a), and water-redispersed CNFs (b), CNF-CMC-HT-10 (c), CNF-CMC-HT-20 (d), and CNF-CMC-HT-100 (e) samples. The samples were observed at x2000 (a, b, c, d, and e) and x20000 (a’, d’, and e’) magnification.

The rheological properties of the water-redispersed samples were examined by measuring the viscosity of 0.5 wt% suspensions as a function of the shear rate. As shown in Figure 10, the viscosity of all the samples showed a non-Newtonian behavior, typical of CNF suspensions. The decrease of viscosity when increasing the shear rate is caused by the shear-induced alignment of the particles in suspension. The viscosity of the redispersed samples increased with increasing adsorbed amount of CMC, reaching values very similar to those of never dried CNFs for the sample CNF-CMC-HT-100. The amount of CMC added in order to recover the viscosity of a never dried CNF suspension by the adsorption process...
at HT (i.e. 100 mg CMC/g CNFs) is three times lower than the previously reported value of CMC needed for the complete recovery of viscosity in suspensions of water-redispersed CNF-CMC composites.\textsuperscript{118}

The mechanical properties of nanopapers prepared from redispersed CNF-CMC suspensions were compared to those of never dried CNFs. Typical strain-stress curves for the different samples recorded at uniaxial tensile testing are plotted in Figure 11. In addition, the mechanical properties obtained from these measurements are summarized in Table 2. The elastic modulus of all the redispersed samples remained similar to that of never dried CNFs. Nevertheless, due to aggregation of CNFs during drying, the strain-to-break and tensile strength of water-redispersed CNFs (4.5% and 126 MPa, respectively) were much lower than those of never dried CNFs (7.4% and 198 MPa). Redispersed samples adsorbed with CMC (CNF-CMC-HT-50 and CNF-CMC-HT-100) exhibited mechanical properties very similar to those of never dried CNFs, confirming the good redispersibility of the CNFs adsorbed with CMC. The strain-to-break of redispersed CNF-CMC samples slightly increased compared to that of the never dried control. This might be cause by the charge introduced at the surface of the CNFs by the adsorbed CMC. The negative charge causes repulsion between the nanofibrils and aids the sliding and rearrangement of CNFs during plastic deformation, in a similar manner as it occurs for carboxylated CNFs.\textsuperscript{60}
Figure 11. Representative stress-strain curves of nanopapers prepared from suspensions of never dried CNFs, and water-redispersed CNFs, CNF-CMC-HT-50, and CNF-CMC-HT-100.

Table 2. Mechanical properties of nanopapers prepared from suspensions of never dried CNFs and water-redispersed suspensions of CNFs, CNF-CMC-HT-50, and CNF-CMC-HT-100.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Strain-to-break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNFs never dried</td>
<td>11.4 ± 0.2</td>
<td>197.9 ± 6.1</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>CNFs dried</td>
<td>10.3 ± 0.6</td>
<td>125.5 ± 8.3</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>CNF-CMC-HT-50</td>
<td>10.0 ± 0.3</td>
<td>189.8 ± 7.8</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>CNF-CMC-HT-100</td>
<td>10.5 ± 0.3</td>
<td>190.8 ± 12.9</td>
<td>7.9 ± 0.8</td>
</tr>
</tbody>
</table>

3.2. Surface quaternized CNFs (Paper II)

Water is the most precious resource on Earth and essential for life. Extensive and careful wastewater management is necessary to keep the planet’s water reserves in good condition. Because of its high impact on the environment and society, wastewater treatment is a high-priority field of research. One sustainable approach to wastewater treatment is the utilization of renewable resources as adsorbents. Materials such as low-cost agricultural waste, shell-fish by-products, and wood have
been investigated. Due to the negative charge present at the surface of carboxylated CNFs, these nanofibrils have been used in the purification of water to remove contaminants such as cationic dyes or radioactive ions. Synthetic dyes are widely used in a large variety of industries. Cationic cellulose fibers have already been studied for their ability to remove anionic dyes, thus cationic CNFs, possessing extremely large surface areas, have great potential in this field of application. Therefore, in Paper II CNFs were prepared by quaternization of pulp fibers followed by a mild mechanical treatment. The cationic charge of the nanofibrils was tailored by optimizing the reaction conditions. Morphology and physicochemical properties of the resulting covalently modified cationic CNFs were characterized. Furthermore, the capacity of quaternized CNFs to adsorb anionic dyes was studied.

3.2.1. Surface quaternized CNFs prepared by covalent modification of pulp fibers

Previous attempts to prepare quaternized CNFs and CNCs led to low DS values, 0.08 and 0.02 mol mol⁻¹, respectively. In those studies, an increase of the glycidyltrimethylammonium chloride used in the reaction caused the destruction of the crystalline structure of CNFs, under conditions of a rather low alkali concentration. In our study, we focused on the optimization of the alkali concentration for a complete activation of the hydroxyls on the surface of CNFs. An excessive concentration of NaOH during the reaction would lead to the dissolution of cellulose and the conversion from cellulose I to cellulose II. For a pulp consistency of 7.5 wt% in water, an optimum concentration of 5 wt% NaOH was established. Thus, by varying of the amount of glycidyltrimethylammonium chloride added to the reaction, the trimethylammonium chloride content of the modified pulp fibers could be tuned from 0.59 to 2.31 mmol g⁻¹, as measured by conductimetric titration (i.e. DS from 0.10 to 0.37).

The covalent coupling of trimethylammonium chloride groups was confirmed by FT-IR. As shown in Figure 12a, a new peak at 1480 cm⁻¹ corresponding to trimethyl groups was present in all the quaternized pulp fibers. Moreover, the intensity of the peak at 1640 cm⁻¹ also increased with the increase of the trimethylammonium groups in the sample. This peak was assigned to the absorbed water in the sample. Therefore, the quaternization of the cellulosic surface increases the hydrophilicity of the sample.
To study the effect of covalent modifications to crystalline structure of the surface quaternized CNFs, all samples were investigated by XRD. As shown in Figure 12b, all the samples show typical diffractograms of the cellulose I crystalline structure, confirming the topochemical character of the modification. After curve fitting, the crystalline and amorphous contributions in the diffractograms were separated. For each sample, two peaks centered at angles of about 14.8 ° and 16.8 ° were observed. These peaks correspond to d-spacings of 0.60-0.61 and 0.53-0.54 nm, respectively. An average crystal size ($C_A$) was determined from the crystal size of the two crystallographic planes calculated by Scherrer’s equation (Eq. 3). From the $C_A$ value, by assuming that cellulose I nanofibrils have square cross-section, an estimation of the hydroxyl groups (mol mol$^{-1}$ bulk AGU) exposed on the surface can be calculated using the following equation:  

$$\text{Exposed surface hydroxyls} = \frac{3 \times (C_A/0.61+C_A/0.53)}{(C_A/0.61+1) \times (C_A/0.53+1)}$$  

Equation 4

Exposed hydroxyl groups calculated for each sample are summarized in Table 3. Estimation of exposed hydroxyl groups for the sample with the maximum trimethylammonium chloride content (i.e. 2.31 mmol g$^{-1}$, DS of 0.37 mol mol$^{-1}$ AGU unit) was 1.07 mol mol$^{-1}$, thus 35% of the exposed hydroxyl groups had been covalently modified to trimethylammonium groups. With 1.5 exposed surface hydroxyl groups per AGU unit, a 35% degree of substitution would result in at
least one trimethylammonium chloride group every other surface AGU, assuming a homogeneous topochemical reaction.

Furthermore, the crystallinity index (CI), i.e. ratio between the area of the crystalline peak and the total reflection area, was also calculated. All the results are summarized in Table 3. The average crystal size shows a decrease with increasing trimethylammonium chloride content, up to 1.32 mmol g⁻¹. For higher contents of trimethylammonium chloride the crystal size remains constant. The CI index of the samples increases with the trimethylammonium chloride, from 61.0% for non-modified wood pulp to 65.2%. This increase in crystallinity is caused by the solubilization of amorphous cellulose regions during the quaternization reactions.

Table 3. Chemical and physical properties of quaternized CNFs. (CA: average crystal size; CI: crystallinity index)

<table>
<thead>
<tr>
<th>Trimethylammonium chloride content</th>
<th>Quaternized CNF dimensions</th>
<th>CA (nm)</th>
<th>Exposed hydroxyl groups mol mol⁻¹ AGU</th>
<th>CI %</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol g⁻¹</td>
<td>mol mol⁻¹ AGU</td>
<td>Width (nm)</td>
<td>Length (μm)</td>
<td></td>
</tr>
<tr>
<td>Pulp fiber -</td>
<td>-</td>
<td>3.1</td>
<td>0.79</td>
<td>61.0</td>
</tr>
<tr>
<td>CNF samples</td>
<td>0.59</td>
<td>0.10</td>
<td>2.1±0.9</td>
<td>2.0±0.8</td>
</tr>
<tr>
<td>0.79</td>
<td>0.13</td>
<td>2.1±0.5</td>
<td>1.4±0.6</td>
<td>2.1</td>
</tr>
<tr>
<td>1.32</td>
<td>0.21</td>
<td>1.8±0.5</td>
<td>1.3±0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>2.31</td>
<td>0.37</td>
<td>1.6±0.5</td>
<td>1.3±0.5</td>
<td>1.9</td>
</tr>
</tbody>
</table>

The morphology of the quaternized pulp fibers was affected by the introduction of cationic charge at the surface of the nanofibrils. As shown in Figure 13, when observing the covalently modified fibers using phase-contrast microscopy, a characteristic swelling of the fibers also known as the ballooning phenomenon was revealed. The ballooning phenomenon, previously reported for TEMPO-oxidized pulp fibers¹⁰⁵ and the dissolution of pulp fibers in NaOH-water,¹²⁹ was more pronounced when the trimethylammonium chloride content increased, due to repulsion forces between cationic nanofibrils in the fiber.
Figure 13. Phase-contrast optical microscope images of original pulp fibers (a), and quaternized pulp fibers with trimethylammonium chloride contents of 0.59 (b) and 0.79 mmol g⁻¹ (c). FE-SEM images and photographs of 0.2 wt% suspensions of unmodified pulp fibers (d), and quaternized CNFs with trimethylammonium chloride contents of 0.59 (e) and 0.79 mmol g⁻¹ (f), and the sample further diluted at 0.1 wt% observed between cross-polarizer (g).

The quaternized pulp fibers were homogenized to prepare suspensions of individualized surface quaternized CNFs. Compared to the original pulp fibers that quickly precipitated (Figure 13d), the homogenized suspension was already stable at low trimethylammonium chloride content (i.e. 0.59 mmol g⁻¹). The freeze-dried sample after solvent exchange was observed using FE-SEM (Figure 13e). The suspension consisted of individualized CNFs and some fibril aggregates. An increase of the trimethylammonium chloride content to 0.79 mmol g⁻¹ led to an optically transparent gel-like suspension (Figure 13f). When placed at rest between cross polarizers, the sample showed frozen-in birefringence, indicating the formation of a well individualized colloidal suspension. The sample exhibited a fine structure of individualized nanofibrils as revealed by FE-SEM, with a more homogenous nanofibril width than that of the sample with lower trimethylammonium chloride content.
The morphology and size distribution of the quaternized CNFs was further studied by topographic imaging with AFM (Figure 14). The average width and length of all the samples are summarized in Table 3. Initially, the increase in trimethylammonium chloride content from 0.59 to 1.32 mmol g\(^{-1}\) caused a decrease in the size of nanofibrils, from 2.1±0.9 to 1.8±0.5 nm in width and from 2.0±0.8 to 1.3±0.5 \(\mu\)m in length. A further increase in the trimethylammonium chloride to 2.31 mmol g\(^{-1}\) did not affect the size. These results are in good agreement with size calculated from XRD experiments.

Quaternized CNFs with different trimethylammonium chloride contents were used to prepare nanopapers. As summarized in Table 4, the resulting nanopapers possessed high porosity due to the repulsion forces between cationically charged nanofibrils. Thus, the porosity in the nanopapers increased with the increase of trimethylammonium content. Despite their high porosity, the nanopapers exhibited remarkable mechanical properties. Modulus and tensile strength of the nanopapers decreased when increasing the trimethylammonium chloride content, due to the increase in porosity in the sample. Regardless of having almost twice the porosity compared to nanopapers prepared from enzymatic CNFs, nanopaper with a trimethylammonium chloride content of 1.32 mmol g\(^{-1}\) showed very similar mechanical properties.
Table 4. Mechanical properties and porosity of quaternized CNF nanopapers.

<table>
<thead>
<tr>
<th>Trimethylammonium content (mmol g⁻¹)</th>
<th>Modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Strain-to-break (%)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaternized CNF nanopapers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.59</td>
<td>10.0 ± 0.2</td>
<td>226.8 ± 20.6</td>
<td>5.2 ± 0.6</td>
<td>37</td>
</tr>
<tr>
<td>0.79</td>
<td>9.9 ± 0.4</td>
<td>187.9 ± 10.5</td>
<td>4.0 ± 0.2</td>
<td>42</td>
</tr>
<tr>
<td>1.32</td>
<td>9.9 ± 0.1</td>
<td>208.7 ± 8.7</td>
<td>4.9 ± 0.6</td>
<td>47</td>
</tr>
<tr>
<td>2.31</td>
<td>9.1 ± 0.4</td>
<td>191.4 ± 6.4</td>
<td>5.5 ± 0.2</td>
<td>48</td>
</tr>
</tbody>
</table>

3.2.2. Capacity of surface quaternized CNFs to absorb water and remove anionic dyes

The nanopapers prepared from quaternized CNFs showed typical porous structure of cellulose nanopapers, with a surface of visible nanofibrils with random-in-plane orientation (Figure 15b) and a cross-section that exhibits a layered structure of quaternized CNF sheets (Figure 15c). When soaked in water, the nanopapers swelled considerably, forming stable hydrogels. As shown in Figure 15d and 15e, this swelling resulted from a separation of sheets that formed the layered structure of the nanopaper. The separation between layers in the hydrogel increased with the increase of trimethylammonium chloride content. Moreover, the formation of a network of individualized quaternized CNF between layers was observed. This indicates that the charge density at the surface of the quaternized CNFs dictates the water absorbency of the nanopaper. The water absorbency was further characterized with a kinetic study. Figure 15a shows the water absorbency of cellulose nanopapers as a function of trimethylammonium chloride content and time. The water absorbency of the nanopapers is strongly dependent on charge density, and the adsorption takes place mostly during the first 24 hours. Compared to nanopaper from non-charged CNFs, which adsorbed only 2 g of water per gram of cellulose, quaternized CNF nanopapers absorbed from 100 to 750 g per gram of cellulose, depending on the trimethylammonium chloride content of the sample.
Figure 15. (a) Water absorbency of quaternized CNF nanopapers and FE-SEM images of the surface (b) and cross-section (c) of quaternized CNF nanopaper with a trimethylammonium chloride content of 1.32 mmol g$^{-1}$, and the swollen cross-sections of CNF nanopapers with trimethylammonium chloride content of 0.59 mmol g$^{-1}$ (d) and 1.32 mmol g$^{-1}$ (e) after 10 hours in deionized water.

The capacity of the different surface quaternized CNF samples to adsorb divalent anionic dyes was tested by adding freeze-dried quaternized CNFs to solutions of congo red or acid green 25. Due to the high specific surface area of the freeze-dried nanofibrils, the anionic dyes are adsorbed in a few tens of seconds. The dye adsorbed CNFs can be easily removed by centrifugation or filtration. The adsorption capacity for congo red and acid green 25 as a function of the trimethylammonium chloride content are shown in Figure 16. The amounts of congo red and acid green adsorbed at each concentration are very similar because of their similar molecular weights. The amount of dye adsorbed increased with increasing the trimethylammonium chloride content up to a content of 1.32 mmol g$^{-1}$; a further increase to 2.31 mmol g$^{-1}$ did not increase the binding capacity. The CNFs with a trimethylammonium chloride content of 1.32 mmol g$^{-1}$ could adsorb 0.664 g g$^{-1}$ of congo red and 0.683 g g$^{-1}$ of acid green 25. These values correspond approximately to 1 mol of dye per kg of quaternized CNFs. This absorbance capacity is similar to those of quaternized cellulose derivatives (Whatman QA52, 1.1 eq. kg$^{-1}$), quaternized sugar cane bagasse and polyamide-epichlorohydrin-cellulose (1.0 mol kg$^{-1}$ of congo II or direct blue 86, respectively).
Figure 16. Adsorbed dye as a function of the trimethylammonium chloride content. Congo red and acid green 25 solutions before and after the addition of quaternized CNFs.

The dye absorbance capacity of quaternized CNF nanopapers was also investigated. Due to their lower specific surface area, dye adsorption with nanopaper was slower than with freeze-dried quaternized CNFs. As shown in Figure 17, when a nanopaper piece (30 mg) was introduced into a 0.5 mg mL\(^{-1}\) congo red solution (35 mL), 75% of the dye was adsorbed in 24 hours. After 72 hours the suspension had almost reached equilibrium (95% removal). Interestingly, the nanopaper preserves its integrity after swelling. Thus, the resulting hydrogel from the clear solution can be easily removed.

Figure 17. Time-dependent absorption spectra of a congo red solution containing quaternized CNF nanopaper with trimethylammonium chloride content of 1.32 mm g\(^{-1}\).
3.3. CNFs grafted with PEG (CNF-g-PEG) (Paper III)

Tailoring the nanostructure of CNF-based materials has a drastic impact on the properties of the resulting materials. A preferential orientation of CNFs in materials such as nanopapers and wet extruded fibers has significantly improved the mechanical properties of these materials. Because of the high aspect ratio of CNFs and the numerous entanglements present in CNF networks, these networks are very difficult to orient. A soft coating at the surface of CNFs can act as a lubricant and facilitate fibril-fibril sliding. Previous studies have shown the decrease in friction between cellulose surfaces adsorbed with CMC grafted with PEG. Moreover, PEG has some other interesting properties. For example, the adsorption of PEG on CNFs via ionic bonds has shown to allow their dispersibility in organic solvents and to improve the interaction between CNFs and poly(L-lactide) in nanocomposites. In addition, PEG coatings prevent protein adsorption and cell adhesion, showing potential use as materials for biomedical devices. Therefore, in Paper III PEG was chosen as a lubrication coating to facilitate the sliding of nanofibrils during shear-induced orientation of CNF networks.

3.3.1. Grafting of PEG onto CNFs

CNF-g-PEG samples were prepared by a three-step process: oxidation of wood pulp, amidation, and disintegration. The amidation reaction was performed at two different temperatures, 22 and 35 °C. The successful covalent grafting of PEG onto CNFs was confirmed by FT-IR. As shown in Figure 18, the TEMPO-oxidized CNFs showed a very clear band at 1729 cm⁻¹, which corresponded to the stretching frequency of the acidic carbonyl groups introduced at the surface of the CNFs by the TEMPO-mediated oxidation. The intensity of this band decreased for the CNF-g-PEG samples due to the consumption of carboxyl groups in the amidation reaction, and was almost not visible for the CNF-g-PEG sample prepared at 35 °C. At the same time, a band for the NH bending (δ_{NH}, amide II) at 1558 cm⁻¹, and bands at 1659 cm⁻¹ and 1608 cm⁻¹ corresponding to the carbonyl stretching (ν_{C=O}, amide I) appeared in the CNF-g-PEG samples. Even though the bands overlapped with the water adsorption band at 1638 cm⁻¹, these bands had higher intensity for the CNF-g-PEG prepared at 35 °C, and confirmed the successful covalent grafting of PEG onto the CNFs.
The reaction performed at each temperature led to very different carboxylate contents in the CNF-g-PEG samples, of 1.1 mmol g\(^{-1}\) at 22 °C and 0.5 mmol g\(^{-1}\) for at 35 °C, as measured by conductimetric titration. Considering the PEG contribution to the weight of the sample, these values correspond to a carboxylate content per gram of CNFs of 1.325 and 0.8 mmol g\(^{-1}\) for the sample prepared at 22 and 35 °C, respectively. Subtracting these values from the carboxylate content of the TEMPO-oxidized pulp fibers (1.6 mmol g\(^{-1}\)), a decrease of 17% and 50% was calculated for the amidation reactions at 22 and 35°C, respectively. Therefore, the reaction at higher temperature showed a higher yield. The dependence of PEG-grafting density on the temperature was previously reported by Kingshott et al.\(^{133}\) As the reaction temperature approaches the cloud point of PEG in water (i.e. 60 °C), the hydrodynamic volume and the radius of gyration of individual PEG chains decrease, resulting in a denser packing of grafted PEG chains on the surface. Previous references have reported the existence of side reactions during the carbodiimide-mediated amidation. These side reactions cause the consumption of carboxyl groups and the formation of undesired N-acylurea.\(^{134, 135}\) Therefore, in

Figure 18. FT-IR patterns of CNF-g-PEG samples (a and b), TEMPO-oxidized CNFs (c), original wood pulp (d), and PEG-NH\(_2\) (e).
order to do not overestimate the amount of PEG covalently grafted to the CNFs, the amount of PEG in the CNF-g-PEG samples was further characterized by TGA.

Figure 19. (a) Thermogravimetric curves for the decomposition of PEG-NH$_2$, TEMPO-oxidized CNFs and CNF-g-PEG samples, and their corresponding 1$^{\text{st}}$ derivative (DTG) weight loss curves (b).

As shown in Figure 19a, the decomposition of a pure PEG-NH$_2$ follows a single-step reaction, with a drastic weight loss between 300 to 450 °C. The decomposition finishes around 450 °C, with only 3.5 % of the initial weight as solid residue. The thermal degradation of TEMPO-oxidized CNFs starts at 210 °C, being this starting decomposition temperature typical for chemically modified cellulose.$^{136, 137}$ As shown in Figure 19b, the decomposition of TEMPO-oxidized CNFs follows a two-step reaction, the 1$^{\text{st}}$ derivative of the weight loss exhibits two clear peaks with maximums at 240 and 300 °C. The first peak is caused by the decomposition of carboxylated cellulose at the surface of the CNFs, while the second peak is caused by the decomposition of the non-carboxylated core of the nanofibrils. After 350 °C, a final slow charring process takes place. The TGA analysis of CNF-g-PEG samples showed a three-step decomposition, which corresponds to the overlapping of the TEMPO-oxidized CNFs and PEG-NH$_2$ decompositions. The two peaks corresponding to the cellulose decomposition for these samples were slightly shifted towards higher temperatures, and this shift was larger for the sample prepared at 35 °C. This phenomenon is possibly caused by the formation of a PEG layer on the surface of the CNFs, which protects the cellulose from degradation, in a similar manner as previously reported for TEMPO-oxidized CNFs grafted with stearylamine.$^{138}$ The decrease in weight corresponding to PEG in the CNF-g-PEG
samples was determined as 12.5% and 32.1% by extrapolation of the PEG component (dotted line in Figure 19a). By assuming that the grafted PEG decomposes as the pure PEG-NH₂, the weight fraction of grafted PEG was 13% and 33% in the CNF-g-PEG samples obtained from reactions at 22 and 35 °C, respectively. Consequently, the two CNF-g-PEG samples were coded as CNF-g-PEG-13 and CNF-g-PEG-33.

The crystallinity of CNF-g-PEG samples was investigated by XRD. As shown in Figure 20, the grafted samples did not show any of the two diffraction peaks at 19.5° and 23.7° of the PEG-NH₂ control. Therefore, the PEG grafted at the CNF surface did not crystallize, possibly due to the short length of the grafted brushes and a homogenous distribution of the PEG along the surface of the nanofibrils. CNF-g-PEG samples showed the typical diffraction profile of cellulose I, very similar to that for TEMPO-oxidized CNFs. Thus, the crystalline structure of the CNFs was preserved after the grafting of PEG. A broadening of the peaks at 16 ° ((110) and (110) planes) and 22.6 ° ((200) plane) was caused by the contribution of the grafted PEG to the amorphous fraction of the samples. The CI of the CNF-g-PEG samples decreased with increasing grafted amount of PEG, with values of 79%, 67%, and 60% for the TEMPO-oxidized CNFs, CNF-g-PEG-13, and CNF-g-PEG-33 samples, respectively.

![Figure 20. XRD diffractograms of PEG-NH₂, TEMPO-oxidized CNFs and CNF-g-PEG samples.](image-url)
The morphology of the CNF-g-PEG samples and a TEMPO-oxidized CNF control was studied by TEM. As shown in Figure 21, all the samples exhibited well individualized CNFs. A remarkable CNF width increase for the CNF-g-PEG-33 sample (5.1 ± 0.6 nm) was evident when compared to the TEMPO-oxidized CNF control (3.7 ± 0.5 nm). The sample CNF-g-PEG-13, with lower content of PEG, showed a heterogeneous width along the nanofibrils length. This is probably caused by an insufficient grafting density of PEG that do not cover the entire surface of the nanofibrils. Assuming a cylindrical nanofibril shape with a diameter of 3.7 nm and a density for cellulose of 1.5 g cm⁻¹, the grafting density of PEG at the surface of the nanofibrils can be calculated. The grafting density of PEG was 0.17 chain/nm² for CNF-g-PEG-13, and 0.56 chains/nm² for CNF-g-PEG-33. Unsworth et al.¹³⁹ reported that a grafting density of 0.24 chains/nm² was necessary to form a monolayer of unperturbed random coils when grafting PEG (Mₚ=750) onto gold surfaces. Therefore, the grafting density in the CNF-g-PEG-13 sample was not sufficient to coat the nanofibrils, while the PEG content in CNF-g-PEG-33 sample is enough for complete coverage of the nanofibrils surface, as observed by TEM. TEMPO-oxidized CNFs present a carboxyl density at their surface of 1.34 groups/nm² thus, for the sample CNF-g-PEG-33 42% of the carboxyl groups were consumed for the grafting of PEG.

![Figure 21. TEM micrographs of TEMPO-oxidized CNFs (a), CNF-g-PEG-13 (b), CNF-g-PEG-33 (c) of PEG.](image)

**3.3.2. Oriented CNF-g-PEG ribbons, mechanical and optical properties**

Hydrogels with solid contents between 80 to 85% were prepared by vacuum filtration of CNF-g-PEG water suspensions. The hydrogels were cut in 5 mm wide strips and stretched 40%, from 50 mm to a final length of 70 mm. The stretching of the samples caused a decrease in width of 3 mm. After stretching, the samples were rapidly dried to preserve their structure. The resulting ribbons had a thickness
of about 70 μm, larger than that of non-stretched ribbons (about 40 μm). The surface morphology of the samples was studied by AFM. As shown in Figure 22a, and 22c, the non-stretched samples (-N) showed the characteristic random-in-plane nanofibrils network of CNF nanopapers. As already observed by TEM, the CNF-g-PEG-33 nanofibrils were considerably wider than the TEMPO-oxidized CNF control. In contrast, with the stretched ribbons (CNF-S and CNF-g-PEG-33-S, Figure 22b and 22d, respectively) showed a rather smooth surface, and the nanofibrils network at the surface of the ribbon was no longer evident. The stretched samples showed a preferential alignment of nanofibrils and nanofibril aggregates along the drawing direction. This alignment was more obvious when larger areas of the ribbon surface were scanned. The preferential orientation was more evident for the CNF-g-PEG-33-S sample, and higher than the alignment observed in previously reported oriented TEMPO-oxidized CNF nanopapers, where the network structure of the surface was still visible. The higher orientation achieved for PEG-grafted samples is due to the lubrication effect of the PEG coating, that aids the sliding and rearrangement of the nanofibrils during drawing.

**Figure 22.** AFM topographic images of the surface of CNF-N (a), CNF-S and zoom-out image (b), CNP-PEG-33-N (c), and d) CNP-PEG-33-S and zoom-out image (d) of non-stretched (-N) and stretched (-S) ribbon samples.
The degree of orientation of the CNFs in the stretched ribbons was further characterized by WAXD. The 2D-diffraction patterns recorded for TEMPO-oxidized CNFs, CNF-g-PEG-13 and CNF-g-PEG-33 ribbons are shown in Figure 23. The diffractograms were recorded with the X-ray beam perpendicular (surface plane, Figure 23a, 23b, 23c, and 23d) or parallel (cross-sectional plane, Figure 23a’, 23b’, 23c’, and 23d’) to the surface of the ribbon. The cellulose crystals in nanofibrils are arranged in the direction of the fiber axis thus, the orientation of the cellulose crystals in a sample can be used to evaluate the orientation of the CNFs. The surface of a non-stretched ribbon control (CNF-g-PEG-33-N, Figure 23a’) showed the typical ring patterns of cellulose I, with three reflection patterns that corresponded to (110), (200), and (004) crystal planes, indicating a random orientation of the nanofibrils in the surface plane. In the cross-sectional plane (Figure 23a), the non-stretched sample showed equatorial arcs that corresponded to (110) and (200) diffractions. A meridian arc corresponding to the (004) diffraction was also observed. These arcs are cause by the alignment of CNFs and the formation of a layered structure in the cross-sectional plane of the sample that occurs during the hydrogel preparation by vacuum filtration. The stretched samples showed alignment in both, surface and cross-sectional planes. The drawing-induced orientation in the surface plane of CNF-S, CNF-g-PEG-13-S and CNF-g-PEG-33-S was evidenced by the presence of the same equatorial and meridian arcs shown for the cross-sectional planes.
Figure 23. XRD diffractograms for CNF-g-PEG-33-N (a), CNF-S (b), CNF-g-PEG-13-S (c), CNF-g-PEG-33-S (d) ribbons recorded with the X-ray beam perpendicular to the cross-sectional (a, b, c, d) or to the surface plane (a’, b’, c’, d’).
To quantify the orientation of the nanofibrils in the ribbons, the orientation index of the cellulose crystals ($f_c$) was calculated by the following equation:

$$f_c = \frac{180° - FWHM}{180°}$$  \hspace{1cm} \text{Equation 5}

Where FWHM is the full width at half maximum of the azimuthal intensity profile distribution of the (200) plane equatorial reflection (Figure 24).

The orientation indices calculated for the surface (SP) and cross-sectional (CS) planes of TEMPO-oxidized CNF and CNF-\(g\)-PEG ribbons are listed in Table 5. A significant increase in the orientation of the cross-sectional plane occurred during stretching. The alignment was more effective in the sample with higher amount of PEG covalently grafted to the CNFs. However, the most remarkable orientation occurred in the surface plane, where the CNF-\(g\)-PEG-33 sample changed from a random-in-plane structure of the non-stretched sample to an orientation index of 0.86 in the stretched ribbon. The orientation in this plane was higher for the CNF-\(g\)-PEG-33-S sample ($f_c=0.86$) compared to the CNF-\(g\)-PEG-13-S sample ($f_c=0.79$) or the CNF-S sample ($f_c=0.77$), evidencing the importance of a homogeneous and complete coverage of the CNF surface with PEG to facilitate the sliding and rearranging of the nanofibrils upon stretching. The beneficial lubricating effect of the PEG coating allowed the achievement of a higher degree of orientation in the surface plane than in previous studies (0.817, and 0.65) where no coating was applied to TEMPO-oxidized CNFs.\textsuperscript{60, 130}
Table 5. Orientation indices of cellulose crystals in CNF-g-PEG ribbons. Density, composition, and mechanical properties of non-stretched (-N) and stretched (-S) TEMPO-oxidized CNF and CNF-g-PEG ribbons.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Orientation index [f]</th>
<th>Density [kg m⁻³]</th>
<th>Volume fraction of CNFs/PEG/voids [%]</th>
<th>Modulus [GPa]</th>
<th>Tensile strength [MPa]</th>
<th>Strain-to-failure [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNF-N</td>
<td></td>
<td>1360 ± 14</td>
<td>90.7 / 0 / 9.3</td>
<td>11.2 ± 0.5</td>
<td>242 ± 10</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>CNF-g-PEG-13-N</td>
<td></td>
<td>1310 ± 12</td>
<td>76.0 / 15.5 / 8.5</td>
<td>8.5 ± 0.5</td>
<td>254 ± 5</td>
<td>9.5 ± 0.6</td>
</tr>
<tr>
<td>CNF-g-PEG -33-N</td>
<td>0.00 0.74</td>
<td>1280 ± 6</td>
<td>57.2 / 38.6 / 4.2</td>
<td>6.4 ± 1.0</td>
<td>220 ± 3</td>
<td>12.3 ± 1.7</td>
</tr>
<tr>
<td>CNF-S</td>
<td>0.77 0.87</td>
<td>1380 ± 10</td>
<td>92.0 / 0 / 8.0</td>
<td>17.2 ± 1.6</td>
<td>388 ± 26</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>CNF-g-PEG-13-S</td>
<td>0.79 0.88</td>
<td>1340 ± 7</td>
<td>77.7 / 16.0 / 6.3</td>
<td>22.1 ± 0.7</td>
<td>487 ± 19</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>CNF-g-PEG-33-S</td>
<td>0.86 0.92</td>
<td>1310 ± 8</td>
<td>58.5 / 39.5 / 2.0</td>
<td>32.3 ± 5.7</td>
<td>576 ± 54</td>
<td>3.2 ± 0.8</td>
</tr>
</tbody>
</table>
Figure 25. Stress-strain curves for TEMPO-oxidized CNF and CNF-g-PEG samples with (-S) or without (-N) stretching.

The mechanical properties of stretched and non-stretched ribbons of CNF-g-PEG and TEMPO-oxidized CNF samples were obtained from the stress-strain curves measured when tensile testing the samples. Typical strain-stress curves and values for modulus, tensile strength, and strain-to-failure of all the samples are summarized in Figure 25 and Table 5, respectively. The strain-to-failure of CNF-g-PEG-N samples increased with increasing grafted amount of PEG, while tensile strength remained largely preserved. The CNF-g-PEG-33-N sample had a strain-to-failure of 12.3 ± 1.7%, twice as high as that of the CNF-N control. This remarkable improvement in ductility was caused by the lubricating effect of the PEG coating on the CNFs, which facilitate the sliding and rearrangement of the nanofibrils during plastic deformation. Nevertheless, elastic modulus and tensile strength decreased with the grafting of PEG, due to the lower volume fraction of CNFs in the samples and the negligible contribution of PEG in the mechanical performance of the material. In contrast, the modulus and tensile strength of the stretched samples increased with the increasing volume fraction of PEG. The modulus and tensile strength of CNF-g-PEG-33-S were 32.3 ± 5.7 GPa and 576 ± 54 MPa, respectively, much higher than those of CNF-S ribbons (17.2 ± 1.6 GPa and 388 ± 26 MPa). Taking in account that the volume fraction of CNFs in CNF-g-PEG-33-S is only 58.5%, the back-calculated modulus corresponding to the CNFs was 55.2 GPa. The stiffness and strength of CNF-g-PEG-33-S ribbons are higher than any other previously reported values for aligned CNF materials, such as nanopapers with a drawing ratio of 1.6 (33 GPa and 400 MPa), filament (18
GPa and 490 MPa), wet-spun fibers (24 GPa, 332 MPa), and wet-stretched CNF fibers with an additional post stretching drawing ratio of 0.28 (34 GPa, 289 MPa).

The CNF-g-PEG ribbons were highly transparent, with transmittances between 85 and 90% for the non-stretched samples. As shown in Figure 26a, the transmittance of the stretched samples was about 80%. This decrease of the transmittance in the stretched samples was possibly caused by the alignment of the nanofibrils in a preferential direction. As shown in Figure 26c, an alignment pattern in the stretching direction was observed for the oriented CNF-g-PEG-33-S sample by optical microscope. This alignment pattern was also observed in electric field aligned CNFs in silicone oil.

![Figure 26](image)

**Figure 26.** (a) Transmittance of TEMPO-oxidized and CNF-g-PEG non-stretched(-N) and stretched (-S) ribbons. Microphotographs of CNF-g-PEG-33-N (b) and CNF-g-PEG-33-S (c) ribbons.

The preferential orientation of the samples had also an impact in their diffuse light scattering behavior. As shown in Figure 27, the light scattered when a laser beam traveled through a non-oriented TEMPO-oxidized CNF ribbon was practically negligible, showing a small and intense spot on the wall. When the same beam traveled through an oriented TEMPO-oxidized CNF ribbon, a stronger scattering was evidenced by an increase of the illuminated region on the wall. Interestingly, the CNF-g-PEG-33-S sample exhibited a larger scattering than the stretched TEMPO-oxidized CNF control. The high orientation and wide nanofibrils found in the CNF-g-PEG-33-S sample resulted in an anisotropic scattering of the laser beam, with an elliptical area illuminated on the wall. The illuminated area was also bigger and more homogeneous in intensity than that for the stretched TEMPO-oxidized CNF sample. This phenomenon demonstrated the structure-properties
relationship between the CNF alignment and the optical behavior of the material. Therefore, it evidences the possibility to tune the light scattering behavior of the CNF-based materials by tailoring the degree of orientation of the CNFs in the material.

Figure 27. Light scattering behavior of non-stretched TEMPO-oxidized CNF ribbon (a) and TEMPO-oxidized CNF (b) and CNF-g-PEG-33 (c) stretched ribbons. Laser point diameter of 1 mm and the distance between the ribbon and the wall 20 cm. The grid on the wall is 1 cm square.

3.4. BC modified with D-ChNCs (Paper IV)

Antibacterial materials are of great interest for biomedical and food packaging applications. Chitosan, the deacetylated derivative of chitin (Figure 28), exhibits very interesting properties such as water-solubility at low pH, good film formation ability, and antibacterial activity. Chitin is the principal component of the exoskeleton of arthropods (e.g. crabs, shrimps and insects) and the cell wall of fungi. Thus, chitin is a greatly abundant biopolymer, and it is widely available as a by-product of the seafood industry. As shown in Figure 28a, chitin possesses a chemical structure that resembles cellulose, with N-acetylglucosamine units linked in a β-1,4 fashion. Thus, this linear molecule of high molecular weight shows similar properties as cellulose, with high crystallinity and self-assembles into nanocrystals or nanofibrils. Chitin nanocrystals (ChNCs)\textsuperscript{109,110} and nanofibers\textsuperscript{142,143} can be isolated from different chitin sources by several routes. Some of these routes might cause partial deacetylation of chitin\textsuperscript{111}. Chitin is biodegradable and biocompatible. Moreover, it has been found to accelerate wound healing.\textsuperscript{144} Chitin nanoparticles can be used to prepare nanostructured materials such as membranes\textsuperscript{145} and hydrogels.\textsuperscript{50} All these remarkable properties make chitin a perfect candidate for biomedical applications.

The motivation of Paper IV was to introduce antibacterial activity into BC, because of great interest of using BC in biomedical applications such as blood vessels or wound dressings. The antibacterial activities of three different kinds of ChNCs were studied for the first time. Partially deacetylated ChNCs (D-ChNCs), which
possessed the highest bactericidal activity, were incorporated into BC network by both an in situ process and post-modification.

![Figure 28. Chitin (a) and its deacetylated derivative chitosan (b).](image)

### 3.4.1. Characterization of A-ChNCs, T-ChNCs and D-ChNCs

Three different types of ChNCs, A-ChNCs, T-ChNCs and D-ChNCs were successfully prepared by acid hydrolysis, TEMPO-mediated oxidation and partial deacetylation, respectively. The crystallinities of all the nanocrystals were studied by XRD. As shown in Figure 29a, the diffractograms of all the samples exhibited peaks at 9.4 °, 19.5 °, 21.0 ° and 23.5 °, typical for α-chitin. Further, the degree of acetylation (DA) of the different ChNCs and the original chitin was determined from solid-state $^{13}$C-NMR measurements (Figure 29b, example of a $^{13}$C-NMR spectrum of D-ChNCs). DA values for all the samples were calculated by dividing the intensity of the resonance signal from the methylated carbon group by that of the C1 of the sugar ring for each sample. The DA values of the ChNCs are summarized in Table 6.

![Figure 29. XRD diffractograms of ChNCs (a) and solid-state $^{13}$C-NMR and X-ray diffraction of D-ChNCs (b).](image)
Table 6. DA, carboxylate content, and average width and length of ChNCs.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DA</th>
<th>Carboxylate content (mmol g⁻¹)</th>
<th>Width (nm)</th>
<th>Length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original chitin</td>
<td>86%</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A-ChNCs</td>
<td>86%</td>
<td>-</td>
<td>9 ± 3</td>
<td>182 ± 91</td>
</tr>
<tr>
<td>T-ChNCs</td>
<td>87%</td>
<td>0.57</td>
<td>6 ± 2</td>
<td>253 ± 110</td>
</tr>
<tr>
<td>D-ChNCs</td>
<td>79%</td>
<td>-</td>
<td>7 ± 3</td>
<td>200 ± 93</td>
</tr>
</tbody>
</table>

The morphology of the different ChNCs was studied by AFM. As shown in Figure 30, all the samples appeared as well individualized nanocrystals with a rod-like shape. The size of the nanocrystals from the different samples was calculated from AFM height images. Average values of width and length of all the samples are summarized in Table 6. A few nanocrystal aggregates, which were formed during the drying of the sample on the mica substrate, were percolated from the calculation.

Figure 30. AFM height images and histograms showing the width and length distributions of A-ChNCs (a), T-ChNCs (b), and D-ChNCs (c).
The antibacterial activity of the different types of ChNCs was tested against \textit{E.coli}. As shown in Figure 31, the D-ChNCs exhibited the highest antibacterial activity, with 99 ± 1\% inhibition of bacterial growth when 5 mg D-ChNC was suspended in liquid medium containing 10^4 cfu of \textit{E.coli} cells and incubated for 3 hours. A-ChNCs and T-ChNCs showed a lower inhibition, 61 ± 15\% and 44 ± 12\%, respectively.

![Figure 31. Antibacterial activity of ChNCs](image)

### 3.4.2. \textit{In situ} modification of BC with D-ChNCs

The addition of D-ChNCs to the growing culture media did not alter the production of BC. Approximately 25\% of D-glucose was converted into cellulose, resulting in yield of 178 ± 8 mg after 14 days of biosynthesis. This yield is very similar to that of control BC sample grown without the addition of nanocrystals. The incorporation of D-ChNCs into BC was confirmed by elemental analysis. The BC/D-ChNC-i sample possessed 8\% of chitin, thus approximately 20\% of the D-ChNC that was added to the growing media was incorporated into the BC network. FT-IR and FE-SEM were employed to further study the distribution of D-ChNCs in the BC pellicle. As shown in Figure 32, the addition of D-ChNCs into the culture led to a modified structure of the BC pellicle. Compared to the homogeneous network of cellulose nanofibers in a BC pellicle (Figure 32a), the top surface of the D-ChNC-modified sample was inhomogeneous. Nevertheless, the effect of the modification was more severe on the bottom surface of the pellicle than on the top (Figure 32c). The bottom surface, which remains in contact with the growing media during the growth of the BC pellicle, presented large aggregates of D-ChCNs attached to the BC nanofibers network. The attachment of the nanocrystals to the BC nanofibers was irreversible, since it resisted the extensive washing of the pellicle at high temperature after incubation. A previous study by
Bianchi et al.\textsuperscript{146} already noted the interaction between cellulose and chitin in dilute solutions and in solid state.

\textbf{Figure 32.} FE-SEM images of the top surface of unmodified BC (a), and the top (b) and bottom (c) surfaces of a BC/D-ChNC pellicle modified \textit{in situ}.

\textbf{Figure 33.} FT-IR spectra of BC (a), top (b) and bottom (c) surface of BC/D-ChNC-i surfaces, and pure D-ChNCs (d).

The modification of the BC pellicle was confirmed by FT-IR. As shown in Figure 33, a new band at 1556 cm\textsuperscript{-1} corresponding to NH bending (\(\delta_{\text{NH}}\), amide II) appeared in the spectra of the modified sample. In addition, the carbonyl stretching (\(\nu_{\text{C=O}}\), amide I) bands at 1658 and 1620 cm\textsuperscript{-1} also appeared in the D-ChCNs
modified sample, although the signal overlapped with the water absorption band at 1640 cm⁻¹. The intensities of the characteristic bands for D-ChNCs were higher in the bottom surface than in the top surface, confirming the inhomogeneous nature of the in situ modification as already observed by FE-SEM.

### 3.4.3. Post-modification of BC nanofibers

To prepare homogeneous BC/D-ChNC nanocomposites with high D-ChNCs content, a post-modification preparation route was utilized. The three-dimensional network structure of BC pellicles was disrupted by high speed blending until a homogenous suspensions was obtained. The suspension of individualized BC nanofibers was then mixed with D-ChNCs in different ratios. The resulting suspensions were filtered and dried to prepare nanopapers (BC/D-ChNC-p) that contained 10, 20, or 50 wt% of chitin. The morphology of the nanopapers was investigated by FE-SEM. Figure 34 shows the surface of the post-modified BC nanopapers. The unmodified BC sample showed a web-like porous structure, with clearly visible BC nanofibers with a width of 50-100 nm. The ends of the nanofibers were not apparent in the images, thus the nanofibers were considered as several micrometers long. Compared to the original BC network (Figure 34a), the porosity of the nanocomposites decreased with higher content of D-ChNCs in the sample (Figure 34b-Figure 34d). This decrease is caused by the higher content of small rod-like nanocrystals that fill the space between the nanofibers. The D-ChNCs, which are thinner than the BC nanofibers, were more apparent in the nanocomposite with higher chitin content (arrows in Figure 34d). The morphology of a nanopaper prepared from pure D-ChNCs (Figure 34e) exhibited a very low apparent porosity, due to the shorter aspect ratio of D-ChNCs compared to BC nanofibers.
3.4.4. BC/D-ChNC nanocomposites, mechanical properties and bactericidal activity

The mechanical properties of air dried BC/D-ChNC-i nanocomposites and BC/D-ChNC-p nanopapers prepared by *in situ* modification and post-modification, respectively, were characterized with uniaxial tensile tests. Representative stress-strain curves for each sample are shown in Figure 35. Moreover, the mechanical properties that were calculated from these experiments are summarized in Table 5.

The tensile strength of pure BC samples showed a drastic decrease between the air-dried pellicle (BC, 449 ± 22 MPa) and the nanopaper (BC/D-ChNC-p 100/0, 193 ± 3 MPa). The decrease in both strength and modulus are caused by the disruption of the continuous three-dimensional network of nanofibers that form the BC pellicle during the nanopaper preparation. Tensile strength and strain-to-break of air-dried pellicles and nanopapers decreased with the incorporation of D-ChNCs. Nevertheless, the network structure of the material is well maintained, even when nanopapers prepared by post-modification contain 50 wt% of D-ChNCs. Possessing twice the tensile strength and four times more elongation-to-break than pure D-ChNC, BC/D-ChNC-p 50/50 retains the toughness of the BC network structure. Even with the considerable loss in strength caused by the disintegration of the continuous BC network, BC/D-ChNC-p samples show a higher strength than previously reported chitin/cellulose composites, which were prepared using...
ionic liquids (tensile strength 34.7 MPa, cellulose/chitin ratio 1/7.6). Furthermore, the mechanical properties of BC/D-ChNC-p nanocomposites are similar to those of nanopapers prepared from cellulose nanofibrils from wood.52

![Figure 35](image.jpg)

**Figure 35.** Typical stress-strain curves for BC/D-ChNC nanocomposites.

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>BC/D-ChNC ratio</th>
<th>Modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Strain-to-break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ modification</td>
<td>100/0</td>
<td>9.3 ± 0.3</td>
<td>449 ± 22</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>92/8</td>
<td>9.2 ± 0.4</td>
<td>377 ± 24</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>Post-modification</td>
<td>100/0</td>
<td>7.4 ± 0.4</td>
<td>193 ± 3</td>
<td>9.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>90/10</td>
<td>7.1 ± 0.2</td>
<td>164 ± 4</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>50/50</td>
<td>7.2 ± 0.1</td>
<td>155 ± 3</td>
<td>8.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0/100</td>
<td>7.3 ± 0.1</td>
<td>89 ± 4</td>
<td>1.8 ± 0.3</td>
</tr>
</tbody>
</table>

The bactericidal activity of BC/D-ChNC nanocomposites (7 mg) was tested against *E. coli* cells suspended in water (3mL of water containing $10^5$ cfu mL$^{-1}$ *E. coli*). As shown in Figure 36, the pure BC control sample showed a bacterial growth of about log 0.5 ± 0.1 after 3 hours incubation. This bacterial growth, even in the absence of added nutrients to the medium, is probably caused by the release of residual nutrients from the dry BC nanocomposites that remain in the sample from the initial cultures of *Acetobacter aceti*. The pure D-ChNC film exhibited a viable *E. coli* cell reduction of log 5. As expected, the bactericidal activity of the BC/D-
ChNC nanocomposites increased with increasing D-ChNCs content in the sample. The nanocomposite with the highest D-ChNCs content, i.e. BC/D-ChNC-p 50/50, showed a viable *E. coli* cell reduction of log 3 ± 0.2 after 3 hours incubation. A reduction of bacterial growth of log 0.9 ± 0.1 and log 0.6 ± 0.1 was recorded for BC/D-ChNC-p 80/20 and 90/10, respectively. Interestingly, regardless of its D-ChNCs content, the bactericidal activity of the *in situ* modified nanocomposite, where the D-ChNC are mainly localized at the bottom surface of the nanocomposite instead of being in the bulk of the nanocomposite. Therefore the concentration of D-ChNC on the surface of the *in situ* nanocomposite is higher than for a homogeneous nanocomposite with similar D-ChNCs content prepared by post-modification. In addition to this bactericidal test, BC/D-ChNC-p 50/50 nanopaper was also tested using initial concentrations of $10^6$ and $10^7$ cfu mL$^{-1}$ *E.coli* cells. After an incubation time of 3 hours BC/D-ChNC-p 50/50 and BC/D-ChNC-i 92/8 nanocomposites showed a reduction of viable cells of log 2.32 ± 0.37 and log 0.67 ± 0.04, respectively. Therefore, BC/D-ChNC nanocomposites exhibit both bactericidal (viable cell reduction > log 1) and bacteriostatic activity.

**Figure 36.** Time-dependent killing efficacy of BC/D-ChNC nanocomposites against *E. coli*. In the experiments, 7 mg of nanocomposite were added to a 3 mL water containing $10^5$ cfu mL$^{-1}$ of *E.coli* cells.
3.5. CNFs from CBM3-transformed tobacco cells (Paper V)

In order to bind to carbohydrates, some enzymes possess special binding domains known as carbohydrate-binding modules. Many of this modules bind to cellulose, thus they are called cellulose-binding modules (CBMs). Moreover, they can selectively bind to amorphous or crystalline cellulose. For example, CBM from family 3 (CBM3) binds to crystalline cellulose. Because of their capacity to bind cellulosic surfaces, CBMs have already been used for surface modification of cellulosic materials such as BC,\textsuperscript{99} or for the crosslinking of pulp fibers in paper production.\textsuperscript{149} Furthermore, the genetic modification of trees to overexpress CBMs during cellulose biosynthesis lead to wood pulp fibers with improved mechanical performance.\textsuperscript{48} In Paper V, the effect of the overexpression of CBM3 on the morphology, structure, and mechanical properties of CNFs from primary cell wall of suspension-cultured tobacco cell was studied.

3.5.1. Isolation of CNFs from CBM3-transformed tobacco cells

To study the effect of CBM3 binding on the CNF surface during cellulose biosynthesis, CNFs were isolated from transgenic tobacco (\textit{Nicotiana tabacum}) cell suspension cultures overexpressing CBM3 (CBM3 line), and compared to CNFs from unmodified tobacco cell suspension cultures (WT line). To isolate the CNFs, the non-cellulosic fraction of the cells was removed by sequential extraction of pectins and hemicelluloses.

The transmittance of dilute suspensions of randomly oriented nanofibrils depends on their cross-sectional area.\textsuperscript{150} Thus, the transmittance of CNF suspensions can be used to qualitatively analyze their fibrillation. As shown in Figure 37, the sonicated suspensions of both tobacco lines showed very high transmittance after only 3 minutes of ultrasonication treatment. The transmittance of the samples was between 95 and 98 \%. These values are very similar to that of homogenous suspensions of TEMPO-oxidized CNFs with a diameter of 3-5 nm isolated from wood pulp.\textsuperscript{11} A longer ultrasonication treatment, of 10 minutes in total, did not lead to any increase in transmittance. Therefore, it was concluded that the samples were completely fibrillated after only 3 minutes of ultrasonication. This treatment is milder than disintegration methods previously used to isolate CNFs from primary cell wall of other sources, such as beet root and potato (15 minutes of blending and 15 passes through a homogenizer),\textsuperscript{22, 55} or fruit tissues (30 minutes of sonication).\textsuperscript{20}
The extracted cellulose suspensions (Figure 38a and Figure 38c) were composed of micrometric particles that still preserved a quite intact cell wall structure. The elongated shape of the cells in the CBM3 line, previously observed by Leijon et al.\textsuperscript{104}, was largely preserved in the extracted cell wall fragments. After only 3 minutes of ultrasonication treatment the cellulose suspensions were already homogeneous and did not precipitate after long storage periods. As shown in Figure 38b and Figure 38d, the sonicated samples showed a web-like structure of well individualized CNFs and some nanofibril aggregates. The aggregation possibly originated during the drying of the sample. No differences were observed between suspensions of the WT and CBM3 lines after ultrasonication.

**Figure 37.** Transmittance of extracted cellulose suspensions from WT and CBM3 tobacco lines after different ultrasonication times.
Figure 38. Freeze-dried 0.1 wt% suspensions of cellulose extracted from WT (a, b) and CBM3 (c, d) tobacco cell suspension cultures before (a, c) and after (b, d) disintegration by 3 minutes of ultrasonication treatment.

3.5.2. Morphology of CNFs from transgenic tobacco

The morphology of the CNFs prepared by ultrasonication of extracted cellulose from tobacco primary cell walls was studied by TEM and AFM. As shown in Figure 39, the TEM analysis of the samples confirmed that CNFs from both WT and CBM3 lines were completely individualized after the ultrasonication treatment, very few bundles of CNFs were observed. No differences in morphology between CNFs from WT and CBM3 lines were obvious. Both CNF samples exhibited a diameter of 4-6 nm. Further, the morphology of the CNFs was also studied by AFM (Figure 39). Similar to TEM analysis, the ends of the CNFs were not apparent, thus the length of CNFs from tobacco primary
cell wall was estimated as several micrometers. The width of CNFs measured by AFM was of 2-3 nm, significantly smaller than the value obtained from TEM analysis. As discussed by Niimura et al., AFM is more accurate than TEM for width determination of CNFs. Moreover, a width of 2-3 nm is in good agreement with previously reported 2-4 nm width of primary cell wall CNFs from sugar beet and potato. This value also agrees with the width of CNFs from celery coelenchyma (2.9-3 nm) measured by small-angle neutron scattering, and the width of R. fruiticosus (2.1 nm) determined by synchrotron x-ray diffraction.

Figure 39. TEM micrographs (a, b) and AFM height images (c, d) of CNFs isolated from WT (a, c) and CBM3 (b, d) lines.

3.5.3. Structural characterization of CNFs

The effect of the transgenic modification on the crystal structure of the CNFs from the CBM3 line was studied. CNFs consist of ordered and disordered regions distributed along the fibril. When the disordered regions of CNFs are removed by a strong acid treatment, the remaining ordered regions that are more resistant to hydrolysis remain. Thus, the length of CNCs is related to the length of the ordered regions found in the native CNFs. Therefore, CNCs were prepared by acid hydrolysis of extracted cellulose from the WT and the CBM3 lines. The yield of the reaction was 11% and 24% for the WT
and the CBM3 line, respectively. Such low yields are probably caused by the low crystallinity of primary cell wall cellulose.\textsuperscript{151} As shown in Figure 40, the resulting CNCs had a coniferous shape and tended to aggregate during drying. The CNCs from the CBM3 line were significantly longer than those prepared from the WT line, with length of 201 nm and 122 nm respectively.

**Figure 40.** TEM images of CNCs prepared by acid hydrolysis of cellulose from WT (a) and CBM3 (b) lines, and their corresponding length distribution histograms.

Since WT and CBM3 lines did not show a significant difference in cellulose crystallinity,\textsuperscript{104} the different size between CNCs from these lines leads us to propose a structural difference between CNFs from the WT and the CBM3 line. Thus, the CBM3 line possesses longer and more widely spaced ordered regions along the CNFs, as schematized in Figure 41.

**Figure 41.** Schematic illustration of the proposed distribution of CNCs along the CNFs from the WT (CNF-WT) and the CBM3 (CNF-CBM3) lines.

### 3.5.4. Mechanical characterization of CNFs from transgenic tobacco

Nanopapers were prepared from water suspensions of individualized CNFs to assess the mechanical performance of CNFs from tobacco primary cell wall. After conditioning at 50 % relative humidity and 22 °C, the nanopapers were tested by
tensile test. The density and mechanical properties of the nanopapers are summarized in Table 8, and stress-strain curves of both samples are shown in Figure 42. The nanopapers, which possessed very similar densities (1.42 and 1.41 g cm\(^{-1}\), for nanopapers from the WT and CBM3 lines, respectively) showed significant differences in their mechanical performance. The nanopaper from the transgenic line was stronger (tensile strength of 198 MPa vs. 143 MPa), stiffer (elastic modulus of 9.3 vs. 8.3 GPa) and more ductile (strain-to-failure of 3.6% vs. 2.3%) than the CNFs from unmodified tobacco primary cell wall. Thus, the CNFs from the transformed tobacco line showed enhanced toughness, with a work-of-fracture of 438 MJ m\(^{-3}\), compared to CNFs from unmodified tobacco primary cell wall (192 MJ m\(^{-3}\)).

![Figure 42. Strain-stress curves of nanopapers prepared with CNFs from the WT (black lines) or CBM3 (red lines) tobacco lines.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Density (g cm(^{-1}))</th>
<th>Modulus (GPa)</th>
<th>Tensile strength (MPa)</th>
<th>Strain-to-failure (%)</th>
<th>Work-of-fracture (MJ m(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.42 ± 0.01</td>
<td>8.3 ± 0.5</td>
<td>143 ± 8</td>
<td>2.3 ± 0.4</td>
<td>192 ± 46</td>
</tr>
<tr>
<td>CBM3</td>
<td>1.41 ± 0.03</td>
<td>9.3 ± 0.2</td>
<td>198 ± 9</td>
<td>3.6 ± 0.4</td>
<td>438 ± 72</td>
</tr>
</tbody>
</table>

**Table 8.** Mechanical properties of nanopapers prepared with CNFs from the WT and CBM3 tobacco lines.
Further, when observed at the tensile-fractured cross-sections the nanopapers showed significant differences. As shown in Figure 43, both samples exhibited the layered structure typical of cellulose nanopapers. This structure was more compact than previously reported images from tensile-fractured surfaces of nanopapers prepared with CNFs from wood pulp. The compact structure agrees with the high density values obtained for the samples. This phenomenon is possibly caused by the lower crystallinity of primary cell wall CNFs, since disordered regions of the CNFs tend to co-crystallize when they come in contact upon drying. Regardless of having the same density prior to tensile test, the fractured sample from the CBM3 line showed a higher porosity, with CNFs pulled out of the surface. This indicates a different deformation mechanism at tensile stress. Because of the altered crystal structure of the transgenic CNFs, with longer ordered regions along the nanofibrils, the nanopaper prepared from modified CNFs was stronger and showed sliding between sheets of the layered structure upon deformation. CNFs were pulled out of the surface of the specimen during fracture, indicating that some shear-induced sliding and rearrangement of CNFs was possible in the modified sample. In contrast, the nanopapers from the WT line showed almost no sliding between CNF sheets and the nanofibrils broke cleanly before being pulled out of the surface.
4. CONCLUSIONS

In this thesis, we have demonstrated how the functionalities and properties of CNFs and CNF-based materials can be tuned by tailoring the surface chemical structure of CNFs through different modification methods, including processing techniques employed during their preparation. Several new routes for the surface modification of CNFs and the applications of the resulting functional materials have been exploited.

By optimizing the conditions for surface modification of CNFs, the functionalization of CNFs can be maximized while preserving their inherent properties. In Paper I, an increase in the temperature from 22 to 121 °C during the process led to a higher adsorption yield of CMC onto CNFs. Thus, the amount of added CMC necessary to achieve a homogeneous coating on the CNFs was significantly reduced. In a similar fashion, the grafting density of PEG chains on the surface of CNFs in Paper III was increased by performing the amidation reaction at higher temperature, in which PEG polymer coils have a relatively smaller radius of gyration. In Paper II, the positive charge density of quaternized CNFs was increased by increasing the concentration of NaOH during the quaternization of wood pulp fibers to activate the surface hydroxyl groups for nucleophilic addition. Furthermore, carrying out covalent modifications on wood pulp fibers prior to their defibrillation was proved to be more efficient than the modification on individual nanofibrils since it allowed reactions with higher concentrations of cellulose.

To achieve a homogeneous surface modification on CNFs was essential for the properties of modified CNF materials. This was highlighted by the achievement of complete water-redispersibility when a monolayer of CMC was adsorbed onto the nanofibrils before drying. In addition, a higher degree of orientation was achieved for CNF-g-PEG ribbons when the chain density of PEG on the surface of the nanofibrils was high enough for a total coverage of the surface.

We have confirmed that the new functionalities introduced to CNFs have led to the fabrication of advanced materials for a broad range of applications. Water-redispersible oven dried CNFs were prepared by the irreversible adsorption of small amounts of CMC (i.e. 2.3 wt%). Nanopapers prepared from highly quaternized CNFs demonstrated high water absorbency (i.e. 750 g g⁻¹). The water absorption capacity of quaternized CNF nanopapers could be tuned by tailoring the content of trimethylammonium chloride groups on the CNFs. Moreover, owing to their
cationic charge, quaternized CNFs were used to remove anionic dye species from water. The functionalization of BC with the incorporation of D-ChNCs produced environmentally friendly nanocomposites that showed bactericidal activity.

We have also shown that tailoring the structure of CNF-based materials has led to enhanced or new properties for the resulting materials. In the modification of BC with D-ChNCs in Paper IV, the *in situ* modification process preserved the native nanofiber network structure of BC and produced nanocomposites with higher mechanical properties than those prepared by the post-modification route. Nevertheless, the direct mixing of individualized BC nanofibers and D-ChNCs in the post-modification route allowed a higher content of D-ChNCs in the nanocomposite and thus achieved higher antibacterial activity. In Paper III, the stretching of CNF-g-PEG or TEMPO-oxidized CNF ribbons induced the alignment of the CNFs along the drawing direction. The aligned samples showed significantly improved elastic modulus and tensile strength, and also an interesting biaxial light scattering behavior. The stretched ribbons of CNF-g-PEG showed a higher degree of orientation compared to the TEMPO-oxidized CNF control, owing to the nanoscale lubrication at the interfaces between CNFs provided by the homogeneous coating of PEG onto the CNFs. The high degree of alignment of the CNFs in CNF-g-PEG ribbons was the main reason for the achievement of the highest mechanical properties reported for oriented CNF-based materials. In Paper V, CNFs from primary cell wall were modified by genetic engineering of suspension-cultured tobacco cells. The transformed cells, that overexpressed CBM3, showed a different structure of the ordered crystal domains along the CNFs. In this CBM3 transformed tobacco line, the ordered regions were significantly longer than those for the WT line. Because of this modified crystal structure in the CNFs, the nanopapers prepared with CNFs from the CBM3 transformed line showed remarkably enhanced toughness.

In conclusion, this thesis work has shown how the optimization of CNF preparation and modification allows the achievement of highly functionalized CNFs with enhanced properties. Furthermore, the influence of the structure of CNF-based materials in their final properties such as mechanical performance or light scattering behavior has also been identified. Herein it has been demonstrated how the modified CNFs can be utilized as building blocks to prepare advanced materials.
5. FUTURE WORK

CNFs possess a great potential as nanoscale build blocks in the preparation of new materials because of their unique properties, high abundance, and sustainable production. In order to promote the use of CNFs industrially and to enhance their use for new applications, further attention focusing on innovative green approaches for CNF modification is needed. For instance, it is necessary to decrease the hydrophilicity of these bio-based building blocks. Thus, the moisture sensitivity of CNF-based materials would be reduced and their compatibility with hydrophobic polymers would be enhanced, resulting in more stable materials and a better reinforcing capacity. Moreover, owing to their biocompatibility, CNFs should be modified to fully utilize their potential for biomedical applications. Finally, it is important to focus the research in CNFs on novel application fields highly necessary for a sustainable society development, such as wastewater treatment or energy production.
6. ACKNOWLEDGMENTS

I wish to thank all the people that made this thesis possible, and that support me during these years in Stockholm. I am very grateful to my supervisor Qi Zhou, without whom this thesis would have never started, for all these years of guidance, fruitful discussions and helpful advice. I wish to thank Lars Berglund, my second supervisor and soul of the division of Biocomposites, for creating such an inspiring work environment and for his deep scientific insight. I also would like to thank Vincent Bulone for adopting me into the division of Glycoscience and for being like a lighthouse in the mysterious sea of biotechnology.

I would like to acknowledge all my collaborators for their hard work. I am especially thankful to Aihua Pei, who was my mentor at my arrival at KTH. She made me feel very welcome and showed me all the basics about biocomposites, and also became a dear friend.

I wish to express my gratitude to my present and former colleagues at the divisions of Biocomposites and Glycoscience for their help and support. Special thanks to Kasinee, for being such a great colleague and a fantastic friend, and to the guys at Albanova for so many terrific memories.

Thank you Skrapan family for making my first months at Stockholm unforgettable. I hope that we can meet again soon! And thanks to all the friends I made playing volleyball at Stockholm for making my time in this beautiful city funnier. Let’s play again soon!!

Gràcies als meus amics de Girona (i comarques!), perquè tot i veure’ns tan poc, quan ens trobem sembla que no hagi passat el temps.

Finalment, m’agradaria agraïr a la meva família tot el seu suport durant tants anys d’estudis, però especialment durant aquest anys en que tot i estar físicament tan lluny, els he sentit sempre molt a prop.
7. REFERENCES


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