Cellulose Nanofibers and Their Assembly for Biomedical and Materials Sciences

Focus on charged cellulose nanofibers
ANNE SKOGBERG

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ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine and Health Technology of Tampere University, for public discussion in the auditorium Pieni Sali 1 of the Festia building, Korkeakoulunkatu 8, Tampere, on 17 November 2023, at 12 o’clock.
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This dissertation research was started in the Micro- and Nanosystems Research Group of Tampere University of Technology Faculty of Automation Science and finalized in the same group of current Tampere University Faculty of Medicine and Health Technology.

This research was made possible by financial support from Graduate School of Advanced Diagnostic Technologies and Applications (DIA-NET), Academy of Finland WoodBone Project and the Centre of Excellence in Body-on-Chip Research.

My path with the dissertation has been long and I'm grateful for meeting and working with inspiring people. First, I am extremely grateful to my supervisor Professor Pasi Kallio for patience, valuable support, guidance and giving me the possibility to work with this unique topic. You were always available to discuss and guide forward. It has been a pleasure to work in the group led by you. I also want to thank the pre-examiners of this thesis, Professor Maria Soledad Peresin and Professor Mehdi Tajvidi.

I am extremely grateful for all my co-authors. I want to thank Panu Lahtinen for providing the nanocellulose materials, it made this research possible. Special thank you goes to Antti Mäki for your valuable contribution with your outstanding data handling skills. Thank you Marja Mettänen for providing your expertise in image analysis. Mari Honkanen, thank you for providing your expertise and guidance in electron microscopy. Markus Hannula, thank you for the micro-CT imaging of the various samples that I have brought to you during the years. Thank you Alexander Efimov for your kind help and experience with NMR studies. I also want to thank all co-supervisors of the papers, Lucie Babakova, T omas Björkqvist and Samp o Tuukkanen. A special thank you for Professor Vesa Hytönen and Rolle Rahikainen for providing the cells used in the first study. I am also grateful for Jenni Leppiniemi for your very first hands-on introduction and advice in nanocellulose research.

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I want to especially thank my co-authors Sanna Siljander and Julia Pajorova for our collaboration and friendship. It has been extremely pleasant to work with you, you both are very talented in your own fields. It has been a pleasure to get to know you both in person.

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Anne Skogberg
Tampere, October 15, 2023
ABSTRACT

Cellulose nanomaterials have novel and improved properties compared to traditional cellulose materials. This combined to the demand for high value-added products and applications made from renewable and sustainable resources makes nanocellulose an appealing material candidate in many fields. In their native state, plant-based cellulose nanofibers (CNFs) are hierarchically aligned. This alignment is lost when individual CNFs are disintegrated from the plant cellulose, and the CNF molecules end up in a gel with entangled shape. Many applications would benefit from materials with aligned structures. Therefore, the alignment of CNF has also been investigated for various purposes, including for advanced biomedical materials and applications. The alignment of CNF is challenging as such, and even more challenging in the presence of other materials.

The main aim of this dissertation was to investigate self-assembly methods for creating aligned and functional CNF and composite film surfaces. The aim was to develop surfaces with aligned CNF and study cell growth and orientation. From skin tissue engineering point of view, the aim was to improve cytocompatibility of a low-cost cellulose mesh using charged CNF coatings and compare cell behavior on anionic (a-) and cationic (c-) CNF coatings. The dissertation also aimed for developing a method to align c-CNF in the presence of multiwall carbon nanotube (MWCNT) component to obtain electrically anisotropic nanocomposite films.

In this thesis, evaporation induced self-assembly was used to align c-CNF along an evaporating boundary line, resulting in surfaces with aligned anisotropic c-CNF. Mouse embryonal fibroblasts were shown to orient and elongate along these aligned CNFs. CNF-driven evaporation-induced assembly was also investigated in the presence of MWCNTs, and this was used to produce nanocomposite films with anisotropic electric conductivity. It was possible to obtain nanocomposite films either with isotropic or anisotropic electrical properties. This was done by careful selection and pretreatment of the nanocomponents for the preparation of the nanocomposite films. Isotropic, evenly conductive films were obtained when high energy sonicated c-CNF/MWCNT dispersion was evaporated. Anisotropic films were formed when additional c-CNF was added to the dispersion inducing c-CNF alignment along the evaporating boundary line.
In this dissertation, cells were cultivated on different CNF surfaces and CNF-coated low-cost cellulose meshes. Mouse embryonal fibroblast proliferation and viability was the highest on a-CNF surfaces. Also, c-CNF surfaces promoted cell proliferation. Human adipose derived stem cell (ADSC) growth was highest on a-CNF coated cellulose meshes. c-CNF coated cellulose meshes induced fast adhesion of ADSCs. However, the viability of ADSCs on c-CNF coated meshes after the 1st day was significantly reduced compared to that of ADSCs on a-CNF and c+a-CNF. Human dermal fibroblast grew well on a-CNF coated and c+a-CNF coated meshes. Their viability on c-CNF coated meshes were poor, although better than on uncoated cellulose meshes.

In conclusion, this thesis showed for the first time that evaporation-induced self-assembly can be used for producing surfaces with aligned CNF, which also promoted cell orientation along the CNF alignment direction. The same CNF driven self-assembly method was used – for the first time – to manufacture anisotropic electrically conductive c-CNF/MWCNT nanocomposite films.
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TIIVISTELMÄ


suuntautuivat näillä pinnoilla järjestäytynneiden nanoselluloosakuitujen suuntaisesti. Nanoselluloosakuitujen järjestäytymistä tutkittiin myös yhdessä hiilinanoputken kanssa, ja niistä valmistettiin sähköisesti anisotrooppisia nanokomposiittikalvoja. Työssä valmistettiin myös tasaisemmin eri suuntaan sähköä johtavia isotrooppisia kalvoja nanoselluloosakuitujen ja hiilinanoputken dispersioista. Anisotrooppiset nanokomposiittikalvojen elinkyky oli heikompi c-CNF pinnoitetuilla verkoilla, ja solujen elinkyky oli kuitenkin a-CNF ja c+a -CNF yhdistelmäpinnoitteisilla alustoilla heikompi. Ihmisperäiset ihon valmistukseen voidaan käyttää nanoselluloosakuitua haihtuvan pisaran rajapinnan suuntaisesti, ja saatiin valmistettua anisotrooppisia nanokomposiittikalvoja.

Tässä työssä solujen kasvua tutkittiin erilaisilla nanoselluloosapinnoilla ja -pinnoitteilla. Hiiren fibroblastisolut kasvoivat parhaiten anionisista nanoselluloosakuiduista (a-CNF) valmistetuilla pinnoilla, joskin myös kationisista nanoselluloosakuiduista (c-CNF) valmistettu pinta oli solujen kasvulle suotuisa alusta. Ihmisen rasvan kantasolut kasvoivat parhaiten a-CNF ja kationisesta ja anionisista nanoselluloosakuiduista (c+a-CNF) valmistetuilla yhdistelmäpinnoitteisilla selluloosaverkoilla ja tarttuivat nopeasti c-CNF pinnoitetulle verkolle. Solujen elinkyky c-CNF pinnoitteisilla alustoilla oli kuitenkin a-CNF ja c+a-CNF yhdistelmäpinnoitteisia alustoja heikompi. Ihmisperäiset ihon fibroblastit kasvoivat hyvin a-CNF ja c+a-CNF pinnoitetuilla verkoilla, mutta niiden elinkyky oli heikko c-CNF pinnoitetuilla verkoilla. Solujen elinkyky oli kuitenkin parempi kaikilla nanoselluloosakuiduilla pinnoitetuilla verkoilla verrattuna pinnoittamattomaan selluloosaverkkoon.

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<tr>
<td>a-CNF(s)</td>
<td>Anionic cellulose nanofiber(s)</td>
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<tr>
<td>ADSC</td>
<td>Adipose derived stem cells</td>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATR-FTIR</td>
<td>Attenuated Total Reflection FTIR</td>
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<tr>
<td>a150</td>
<td>a-CNF coating with volume 150 µL</td>
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<tr>
<td>a600</td>
<td>a-CNF coating with volume 600 µL</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Control sample (Study III)</td>
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<tr>
<td>CCVD</td>
<td>Chemical vapor deposition</td>
</tr>
<tr>
<td>CNC(s)</td>
<td>Cellulose nanocrystal(s)</td>
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<td>CNF(s)</td>
<td>Cellulose nanofiber(s)</td>
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<tr>
<td>c-CNF(s)</td>
<td>Cationic cellulose nanofiber(s)</td>
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<td>CNT</td>
<td>Carbon nanotube</td>
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<td>CV</td>
<td>Circular variance</td>
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<td>c150</td>
<td>c-CNF coating with volume 150 µL</td>
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<td>c600</td>
<td>c-CNF coating with volume 600 µL</td>
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<tr>
<td>c+a</td>
<td>Combination coating of c-CNF and a-CNF</td>
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<tr>
<td>dH₂O</td>
<td>Deionized water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>D₂O</td>
<td>Deuterium oxide, heavy water</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EDC/NHS</td>
<td>1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride/N-hydroxy succinimide</td>
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<tr>
<td>EPTMAC</td>
<td>2,3-epoxypropyl trimethylammonium chloride</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FG-MAS</td>
<td>Field Gradient Magic Angle Spinning</td>
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<td>FN</td>
<td>Fibronectin</td>
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ABBREVIATIONS

FTIR        Fourier Transform Infrared
GTMAC       Glycidyltrimethylammonium chloride
hes          High energy sonicated
hes-c-CNF-MWCNT High energy sonicated cationic CNF multiwalled carbon nanotube
HIM          Helium Ion Microscopy
HPH          High Pressure Homogenization
HPTMA        Hydroxypropyltrimethylammonium
HSC          High spot count
IR           Infrared
IR-imaging   Infrared imaging
ITO          Indium Tin Oxide
MAS-NMR      Magic Angle Spinning Nuclear Magnetic Resonance
Mdn          Median
MEF          Mouse Embryonal Fibroblasts
MFC          Microfibrillated cellulose
micro-CT     X-ray microtomography
MWCNT        Multiwalled Carbon nanotubes
NHDF         Neonatal Normal Human Dermal Fibroblast
NMR          Nuclear Magnetic Resonance
OM           Optical Microscope
PDMS         Polydimethylsiloxane
PLA          Poly(lactic acid)
PS           Polystyrene
PVAm         Polyvinylamine
QAC          Quaternary ammonium compound
rpm          Rounds per minute
RT           Room temperature
SD           Standard deviation
SEM          Scanning Electron Microscopy
SR           Swelling Rratio
(S)TEM       (Secondary) Transmission Electron Microscopy
STT          Surface tension torque
SWCNT        Single-walled carbon nanotubes
S1           Sample 1 (suspension) (study III)
S2           Sample 2 (suspension) (study III)
S3  
Sample 3  (suspension)  (study III)

TEMPO  
2,2,6,6-tetramethylpiperidine-1-oxyl

u-CNF(s)  
Native unmodified cellulose nanofiber(s)

UV  
Ultraviolet light

v/v  
Volume/volume

w/o  
Without

w/w  
Weight/weight

1D  
One dimensional

2D  
Two dimensional

3D  
Three dimensional

\( \alpha^\circ \)  
Location of the positive probe in Measurement D and E

\( F \)  
Force

\( F_l \)  
Surface tension force of the liquid

\( F_s \)  
Surface tension force of the substrate

\( F_{sl} \)  
Interfacial surface tension force

\( I_{avg} \)  
Average current

\( l \)  
Length of the lever arm vector

\( n_a \)  
Location \((n)\) of negative probe

\( p_n \)  
Location \((n)\) of positive probe

\( \Delta R \)  
Swelling ratio

\( R \)  
Resistance

\( R_a \)  
Arithmetic average height parameter

\( U \)  
Voltage

\( W_s \)  
Weight of the swollen sample

\( W_0 \)  
Initial weight

\( \alpha \)  
Contact angle

\( \beta \)  
Angle between the long axis of the affected CNF and the triple line

\( \theta \)  
Angle

\( \lambda_a \)  
Average wavelength

\( \tau \)  
Torque

\( \tau_s \)  
Surface tension torque
ORIGINAL PUBLICATIONS

Publication I  

Publication II  

Publication III  

*Authors contributed equally
AUTHOR’S CONTRIBUTION

Publication I The author designed the study. The author primarily performed the experimental work. The author performed the data analysis with Antti Mäki. Marja Mettänen provided expertise in image-based analysis. Panu Lahtinen contributed to CNF preparation. The author wrote most of the publication. All co-authors participated in the final editing of the manuscript. Pasi Kallio supervised the work.

Publication II The author and Julia Pajorova designed the study. Pajorova carried out the cell experiments and microscopy imaging with help of Broz, Travnickova and Zikmundova. The author fabricated the nanocellulose coatings, the material samples and performed their swelling tests, and contact angle measurements. The author carried out the SEM imaging with Mari Honkanen and Pajorova. Markus Hannula conducted micro-CT imaging. Daniel Hadraba did the AFM scanning and helped the author and Pajorova in analysis. Panu Lahtinen contributed to CNF preparation. The author and Pajorova interpreted the results. The author and Pajorova wrote the publication. All co-authors participated in the final editing of the manuscript. Pasi Kallio and Lucie Bacakova supervised the work.

Publication III The author and Sanna Siljander designed the study. Siljander carried out and optimized the dispersion formation. The author designed and fabricated the films. The author carried out the electrical measurements. The author and Siljander performed IR-imaging and FTIR. The author and Mari Honkanen carried out electron microscopy. Markus Hannula carried out the micro-CT imaging. Alexander Efimov conducted the NMR experiment and helped the author in result interpretation. The author, Siljander and Antti Mäki carried out the data analysis of the IR-imaging. The author and Mäki performed the data analysis of electrical measurements. Panu Lahtinen contributed to CNF preparation. The author and Siljander wrote the publication, as well as analyzed and interpreted the overall results together. All co-authors participated in the final editing of the manuscript. Pasi Kallio, Sampo Tuukkanen and Tomas Björkqvist supervised the work.
1 INTRODUCTION

There is increasing demand for products made from renewable and sustainable resources that are biodegradable, non-petroleum based, carbon-neutral, and have low environmental, animal or human health and safety risks. (Moon et al. 2011; Stoudmann, Nowack, and Som 2019) Wood-derived cellulose materials have been used for thousands of years, and their use is now widespread worldwide and on an industrial scale. Cellulose nanomaterials have novel or improved properties compared to traditional cellulose materials, and the objective is often to develop and produce high value-added products and applications of superior end-use performance (Thakur et al. 2021).

In their native state, plant-based cellulose nanofibers (CNFs) are often aligned into highly hierarchical structures, giving plants their optimal mechanical properties. Engineering applications have various motivations for alignment of CNFs, such as the need for anisotropic properties; adjusting the stiffness and strength of structural materials; optimizing reinforcement efficiency in fiber-reinforced composites; and advancements in biomedical materials and applications. However, despite several attempts and extensive research, realignment of disintegrated and fibrillated CNF remains challenging, so superior CNF properties are often challenging to transfer into optimal macroscale performance. (Li et al. 2021)

A characteristic of cellulose nanomaterials is their tendency to form films upon water evaporation via the strong interactions between the hydroxyl groups on the fibril surfaces (Tammelin and Vartiainen 2014). Since the first reports of the fascinating properties of the CNF films prepared from TEMPO-oxidized CNF (Fukuzumi et al. 2009), nanocellulose-based films have aroused considerable interests in both industry and academia. CNF film applications have extended in value-added fields by the introduction of enhanced performance and/or novel functionalities into the CNF-based films. (Fang et al. 2019). However, although significant progress has been made in strengthening the properties of CNF films, as well as adding novel functionalities, there is an urgent need for simple methods to transfer the superior nanoscale properties of CNF and other functional nanomaterials into macroscale CNF-based films. It would be possible to strengthen
the inherent properties of nanocellulose-based films with careful structural design and/or by combining functional materials with nanocellulose suspensions (Fang et al. 2019). Nanotechnologies have the potential to enhance the utilization of renewable biomaterials and reduce their supply chain costs as well as add functionality. This will make the forestry materials more competitive in high value applications (Hamad 2006), such as in the biomedical field.

One of the aims in the present thesis on a larger scale was to help transfer some of the superior properties of CNFs into macroscale CNF-based substrates. The main aim of this dissertation was to investigate self-assembly methods for creating aligned and functional CNFs and composite film surfaces. The aim was to develop surfaces with aligned CNF and study cell growth and orientation on these aligned surfaces. From a skin tissue engineering point of view, the aim was to improve cytocompatibility of a low-cost cellulose mesh using charged CNF coatings and compare cell behavior on anionic and cationic CNF coatings. The final aim of this dissertation was to develop a method to align cationic CNF (c-CNF) in the presence of a multiwall carbon nanotube (MWCNT) component to obtain electrically anisotropic nanocomposite films.
2 LITERATURE REVIEW

2.1 Cellulose nanofibers

An increasing demand for renewable and sustainable resources favors the use of plant-derived cellulose, which is the most abundant polymer on earth. It is renewable, biodegradable, and nontoxic (Dufresne 2013; Stoudmann et al. 2019; Xue, Mou, and Xiao 2017). Cellulose consists of anhydroglucose units linked together by β-1,4 linkages (Zhang et al. 2013a). These polysaccharide chains are the elementary cellulose chains that are assembled in the form of ordered parallel layers forming elementary fibrils. Such cellulose chains, linked by van der Waals forces and strong intra- and intermolecular hydrogen bonds, form tight aggregates in the plant cell walls (Xue et al. 2017). Described tight cellulose aggregates can be disintegrated into cellulose micro- and nanomaterials, including microfibrillated cellulose (MFC) (Lindström et al. 2014), CNFs and cellulose nanocrystals (CNCs) (Xue et al. 2017; Zhang et al. 2013a).

The manufacture of cellulose nanomaterials from plant fibers includes mechanical, chemical, and enzymatic methods. Fibrillation of wood fibers into smaller CNFs is achieved using mechanical forces, chemical treatments, enzymes, or a combination of these (Thakur et al. 2021). High-pressure homogenization (HPH) is the most widely used mechanical disintegration technique for both lab-scale and industrial scale production of CNF. Other mechanical techniques that are used include microfluidization, micro-grinding, cryocrushing, and ultrasonication (Abdul Khalil et al. 2014; Xue et al. 2017). The drawbacks of mechanical delamination of fibers are high energy consumption and pulp clogging, which can be addressed with certain pretreatments prior to mechanical treatments. Pretreatments aim to weaken the hydrogen bonds, to modify them using repulsive charges (Naderi 2017), or to decrease the degree of polymerization. Such treatments include mechanical refining, alkaline, enzymatic or acid hydrolysis, steam explosion, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation (Isogai, Saito, and Fukuzumi 2011), carboxymethylation, and acetylation (Lavoine et al. 2012; Mondal 2017; Xue et al. 2017). Pre-treatments assist mechanical treatments by facilitating fiber delamination, and thus lower the energy required for fibrillation (Xue et al.
The width of fibrillated CNF is typically between 3 nm and 100 nm, while its length can be several micrometers (Nanotechnologies, ISO 20477 2017). CNFs consist of amorphous and crystalline regions that can be separated using acid hydrolysis, which degrades the amorphous regions, yielding CNCs (Zhang et al. 2013a). After disintegration, the dimensions of CNFs are typically between 3 and 50 nm in cross-section and from 100 nm to several micrometers in length (Nanotechnologies, ISO 20477 2017). Entangled CNFs are longer, while CNCs possess shorter needle- or rod-like morphology with similar diameter and are more rigid due to their higher crystallinity (Habibi, Lucia, and Rojas 2010; Zhang et al. 2013a). As the focus in this thesis is on CNFs, it will be introduced in more detail.

In addition to the abundant available resources, the advantage of wood-derived CNFs is that they can be processed at an industrial scale at a relatively low cost (Lee, Hamid, and Zain 2014; Lin and Dufresne 2014), and produced with a variety of functional groups and using different industrially attractive processes (Naderi 2017). Thus, they can be processed with a variety of surface chemical properties. There are increasing efforts to take advantage of the unique properties of CNFs, such as its nanoscale dimensions, high surface area (high aspect ratio), unique morphology, low density, mechanical strength, barrier properties (Naderi 2017; Zhang et al. 2013a), binder properties (Tayeb et al. 2018), high self-association tendency, swelling ability (Tammelin and Vartiainen 2014), and ability for aligned structures with anisotropic properties (Li et al. 2021). Due to such many advantageous properties, a more widespread use of CNFs is expected in future (Naderi 2017; Zhang et al. 2013a).

CNF have many promising opportunities, specifically, in the development of packaging materials, aerogels, composites, bioactive materials and inorganic/organic hybrid materials (Zhang et al. 2013a), as well as in printed electronics (Hoeng, Denneulin, and Bras 2016), sensor applications (Fu et al. 2021; Salas et al. 2014), catalysis, wastewater treatment, flame retardants (Thakur et al. 2021), binder application (Tayeb et al. 2018) and electrochemical energy storage (Chen et al. 2018). In addition to the increased interest in biomedical applications in general (Bacakova et al. 2019; Thakur et al. 2021; Xue et al. 2017; Zhang et al. 2013a), CNFs are an interesting biological fibrous material for cell culturing applications because of their abundancy, their non-animal biological origin, several chemical modification and functionalization possibilities through the hydroxyl groups on the sugar moieties, their extracellular matrix (ECM) -mimicking fiber dimensions, the possibility for controlled enzymatic degradation (Bhattacharya et al. 2012; Lin and Dufresne 2014; Lou et al. 2014; Zhang et al. 2013a), and the possibility of forming aligned topographies (Li et al. 2021).
A distinguishing characteristic of CNFs is the high degree of hydroxyl groups along the cellulose chain, which enable intra- and inter-molecular hydrogen bonds. The abundance of hydroxyl groups leads to strong affinity to CNF itself and toward materials containing chemical groups, which can form hydrogen bonds with the hydroxyl groups of the cellulose backbone (Moon et al. 2011). Such functional groups include other hydroxyl (–OH), carbonyl (–C=O) and carboxyl groups (–COOH), for example. Fibers in the wood pulp are negatively charged by nature. Anionicity is due to both cellulose and hemicelluloses (Odobas et al. 2016).

What makes CNFs highly versatile and tunable is their different manufacturing techniques and variety of post-treatment possibilities, more commonly referred to CNF modification. This can improve CNF properties and make it more compatible and/or homogeneous dispersion within matrices (Xue et al. 2017). There are a wide range of modifications available for CNFs (Kalia et al. 2014; Thakur et al. 2021). The present dissertation uses the anionic and cationic modifications, which are therefore described in more detail in the following sections.

2.2 Anionic and cationic modifications of cellulose nanofibers

Relevant to this dissertation, two common chemical modifications are introduced that change the charge of the cellulose chain: (1) anionic TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical)-mediated oxidation (Isogai et al. 2011; Saito et al. 2006) and (2) grafting of cationic glycidyltrimethylammonium chloride (GTMAC) (Courtenay et al. 2017), which is also known as 2,3-epoxypropyl trimethylammonium chloride (EPTMAC) (De La Motte et al. 2011; Hua et al. 2015a; Olszewska et al. 2011a). The benefit of these modifications is in controlled adjustment of surface properties of CNFs. Figure 1 presents the modified monomer unit of cellulose backbone after TEMPO oxidation (A) and EPTMAC cationization (B).

2.2.1 Introduction of negative charges through TEMPO-mediated oxidation

Saito et al. (Saito et al. 2006) and Isogai et al. (Isogai et al. 2011) have developed and improved a method to prepare negatively charged cellulose nanofibers from wood cellulose to obtain individual nanofibers 3–4 nm in diameter and at least a few µm in length. They used TEMPO-mediated oxidation, which introduces carboxylate groups at the 6th carbon atom of the glucose unit (Isogai et al. 2011; Saito et al. 2006).
The primary alcohols of cellulose backbones can be converted to carboxylate (COO\(^-\)) functionalities in aqueous environment by a TEMPO-catalyzed reaction in the presence of a primary oxidizing agent, such as sodium hypochlorite (NaOCl) (Kalia et al. 2014).

TEMPO-mediated oxidation allows mechanical disintegration of oxidized fibers using low energy (Isogai et al. 2011; Kalia et al. 2014; Saito et al. 2006). Introduced carboxyl groups can be used to drive the adsorption of other bioactive molecules and further functional groups and enable cross-linking (Weishaupt et al. 2015). Covalent modification of TEMPO-oxidized CNFs can be used to convert carboxyl groups; for example, to amine-reactive via 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) coupling chemistries followed by subsequent conjugation of antibodies and proteins on activated CNFs (Orelma et al. 2012).

### 2.2.2 Properties of TEMPO-oxidized cellulose nanofibers

TEMPO-mediated oxidation pre-treatment significantly decreases the energy consumption as well as the size of the resulting CNF. Compared to the energy demands of HPH, TEMPO-mediated oxidation pre-treatment decreases the energy consumption by a factor of more than 100. This is believed to be partly due to negative charges introduced during oxidation that give rise to repulsive forces between microfibers with the cell wall, causing loosening of microfibril cohesion initially held by hydrogen bonding. Another reason for reduced energy consumption due to more efficient delamination is that the oxidation enhances the hydration and swelling of the fibers, which makes them more flexible and makes their crystalline zone more accessible. In addition, due to loosening of the primary and S1 cell wall during oxidation, the S2 layer becomes more accessible and more prone to fibrillation. However, oxidation creates defects to the fiber cell wall by chain scission in the amorphous zone, which further promotes the mechanical fibrillation (Kalia et al. 2014).

TEMPO-oxidation promotes the formation of remarkable number of C6 carboxylate groups on CNF surfaces (Isogai et al. 2011). A significant reduction in CNF length occurs during the oxidation. The higher the carboxyl content and decrease in size, the better the optical transparency of the resulting CNF suspension. This is due to reduced fiber size with a higher degree of carboxylation, caused by increase in oxidation time (Kalia et al. 2014). The oxidation pre-treatment does not
cause changes to the original crystallinity or crystal width of wood-derived celluloses. Negative-charge-induced electrostatic repulsion and osmotic effect between CNF enables stable dispersion of individual TEMPO-oxidized CNF. According to relatively constant zeta-potential values, all TEMPO-oxidized cellulosics are prone to having almost the same density of carboxylate groups on the surfaces of CNFs, although the carboxylate content may vary depending on the microfiber widths of the native cellulosics (Isogai et al. 2011).

2.2.3 Introduction of positive charges with GTMAC cationization agent

The natural negative charge of cellulose materials can be reversed with a cationization reagent commonly used with both starch and cellulose (Odabas et al. 2016). Such a cationization agent is 2,3-epoxypropyltrimethylammonium chloride (EPTMAC), also known as glycidyltrimethylammonium chloride (GTMAC) (Bendoraitiene et al. 2006; Courtenay et al. 2017; De La Motte et al. 2011; Hua et al. 2015a; Olszewska et al. 2011a). The chemical modification is obtained by reacting a dissolving pulp with one of the cationization reagents (Olszewska et al. 2011a); that is, by grafting the cationic hydroxypropyltrimethylammonium (HPTMA) group to the 2nd carbon atom of the glucose unit (Courtenay, Ramalhete, et al. 2018). The modification of cellulose is done in the presence of varying amounts of water and sodium hydroxide (NaOH). In the chemical reaction, NaOH acts as a nucleophile that activates the hydroxyl groups of the cellulose backbone toward etherification, which allows the grafting of HPTMA (Odabas et al. 2016).

Cationization of the dried softwood pulp facilitates the HPH defibrillation processes and has been shown to prevent clogging of the homogenizer (Chaker and Boufi 2015). Reasons for reduced energy consumption are similar to those described in the case of TEMPO-mediated oxidation. During cationization, quaternary trimethylammonium groups are bound on the surface of microfibers. The presence of these ionic groups leads to an osmotic swelling of the fibers, which reduces hydrogen bonding interaction among the microfibers and increases the distance between neighboring fibers (Chaker and Boufi 2015).
2.2.4 Properties and applications of cationic cellulose nanofibers

Cationic CNFs (c-CNFs) have potential use in both high-end applications such as nanocomposites and high-volume applications in the paper industry (Olszewska et al. 2011a). As cationic materials are generally known to be antiseptic (Gilbert and Moore 2005), incorporating of c-CNFs can give the material antibacterial properties (Chaker and Boufi 2015) in addition to other potential beneficial properties (Olszewska et al. 2011a). The high antibacterial effect of c-CNFs can enhance their use in wound dressings (Chaker and Boufi 2015). It is the quaternary ammonium compound (QAC) that acts as an antibacterial agent (Gilbert and Moore 2005; Zhang et al. 2013a). The QACs are amphoteric surfactants, including one central positively charged quaternary nitrogen associated with four substituents, at least one of which is a major hydrophobic substituent (Gilbert and Moore 2005). At a molecular level, the antibacterial mechanism is based on the interaction of QACs with lipids and proteins that compose the cytoplasmic membrane, involving an association of the positively charged quaternary nitrogen with the head groups of acidic-phospholipids within the membrane, thus damaging the membrane and disturbing the metabolism of bacteria (Chaker and Boufi 2015; Gilbert and Moore 2005).

Cationic CNFs effectively adsorb negatively charges compounds (Voisin et al. 2017) and can be used for ionic crosslinking of two adjacent cellulose chains (Montazer and Harifi 2018). Cationic CNFs also has the potential to bind and release hydrophobic drugs (Jackson et al. 2011). Cationic CNFs can have reinforcing properties in different matrices. However, it has lower reinforcing properties than anionic CNFs, due to the capacity of anionic CNFs to build up stronger networks held by hydrogen bonding (Chaker and Boufi 2015).

![Chemical structure of TEMPO-oxidized (a) and EPTMAC modified (b) CNF subunits.](image)

**Figure 1.** Chemical structure of TEMPO-oxidized (a) and EPTMAC modified (b) CNF subunits.
2.2.5 Anionic and cationic cellulose nanofiber films and coatings

One of the characteristics of cellulose nanomaterials is their tendency to form films upon water evaporation via the strong interactions between the hydroxyl groups on the fibril surfaces (Tammelin and Vartiainen 2014). Since the first reports of the fascinating properties of the CNF films prepared from TEMPO-oxidized CNFs (Fukuzumi et al. 2009), nanocellulose-based films have aroused considerable interest in both industry and academia (Fang et al. 2019). CNF film applications have been extended into value-added fields by enhanced performance and the introduction of novel functionalities into the CNF films (Fang et al. 2019).

The literature reports roughly on two types of CNF films: ultrathin films, also sometimes referred to as model cellulose films (Ahola et al. 2008; Kontturi, Tammelin, and Österberg 2006); and macroscale films, also referred to as self-standing films (Fang et al. 2019). Ultrathin model films – less than 100 nm thick – are often used to study nanoscale behavior at interfaces as they enable the precise molecular level phenomena detection and interpretation (Hakalahti 2018) as well as repeatable and comparable film fabrication, when a model film preparation technique is followed (Kontturi et al. 2006). Ultrathin model films can be prepared using several methods, including spin-coating (Ahola et al. 2008; Kontturi et al. 2006; Olszewska et al. 2011a), Langmuir-Blodgett deposition and layer-by-layer assembly (Shichao Zhang, Xing, and Li 2018). Relevant to the present work, macroscopic films involve the aim to transfer the superior CNF properties to macroscopic films (Fang et al. 2019) and to use them in true applications such as packaging materials, displays, membranes (Hakalahti 2018), electronics, diagnostics, biomedicine, solar cells, and sensors (Tammelin and Vartiainen 2014).

Macroscopic CNF films are self-standing assemblies with a micrometer range thickness (Hakalahti 2018), commonly produced by solvent casting (Aulin, Gällstedt, and Lindström 2010; Österberg et al. 2013; Spence et al. 2010) and filtration-based techniques (Fukuzumi et al. 2009; Isogai et al. 2011). Isogai et al. (Isogai et al. 2011) produced self-standing films from TEMPO-oxidized CNF, with tensile strengths of 200–300 MPa and elastic moduli of 6–7 GPa. Isogai et al. (2011), and Fukuzumi et al. (Fukuzumi et al. 2009; Isogai et al. 2011) reported extremely low oxygen permeability of their TEMPO-oxidized CNF-coated poly(lactic acid) (PLA) films. Both studies reported a surprisingly high decrease in permeability of TEMPO-oxidized CNF-coated PLA compared to PLA alone. Generally, the barrier properties of CNFs are relatively independent of the pre-treatments when a sufficient degree of fibrillation has been achieved. However, charged CNFs with a higher substitution
degree – that is, a higher number of charged groups – tend to absorb more water, which results in enhanced gas permeability (Naderi 2017).

Many studies have reported unmodified and anionically modified CNF film properties, but cationically modified CNFs have attracted less attention in the literature (Olszewska et al. 2011b). Olszewska et al. (Olszewska et al. 2011b) reported pH-dependent swelling behavior of ultrathin cationic CNF films due to the amphoteric nature of the cationized CNFs. Swelling was considerable in all studied pHs, although at pH 8 the swelling degree (that is, the amount of absorbed water molecules) was remarkably insensitive to changes in electrolyte concentration. At pH 4 the film was like a swollen polyelectrolyte gel, but expelled water when electrolyte concentration was increased. At pH 8, the film became remarkably stiffer, likely due to ionic cross-linking (Olszewska et al. 2011b).

Despite significant progress in strengthening the properties of CNF films, as well as adding novel functionalities, there is an urgent need for simple methods of transferring the superior nanoscale properties of CNF and other functional nanomaterials into macroscale CNF-based films. It is possible to strengthen the properties of CNF-based films with careful structural design and/or a combination of functional materials and CNF suspensions. For example, the mechanical properties of a CNF-based film have been improved by aligning the nanocellulose (Fang et al. 2019).

2.3 Water interaction with cellulose nanofibers and the effect of drying

In this dissertation, evaporation-induced self-assembly of CNF water-based suspensions plays a major role. Therefore, water interaction with CNFs (Section 2.3.1), dehydration and hornification phenomena (Section 2.3.2) and the effects of drying on CNFs (Section 2.3.3) are introduced in more detail.

2.3.1 Water interactions

The large surface area of CNF carries a huge amount of hydroxyl groups and therefore has a high affinity towards water. CNF forms hydrogels at low solids content, that is, at less than 2 weight percent (w%) due to their large surface area, high aspect ratio, nanoscale fine structure, their strong tendency to self-associate, as
well as their swelling behavior. If the water in these hydrogels is allowed to evaporate in elevated temperature, for example – the nanofibrillar network forms film-like structures via the strong interactions within many surface hydroxyl groups, as described in Section 2.2.5 (Tammelin and Vartiainen 2014).

TEMPO-oxidized CNF is more moisture sensitive, and thus more hydrophilic and hygroscopic than unmodified CNF due to large amount of carboxylic acid groups on the fibrils. At elevated humidity (≥50 percent room humidity), dissociated carboxylic acid groups are more prone towards water than hydroxyl groups (Tammelin and Vartiainen 2014).

Olszewska et al. (Olszewska et al. 2011a) characterized interactions between c-CNFS and water and described the amphoteric nature of c-CNFS. After the cationization process described in Section 2.2.3, the quaternized nitrogen of the trimethylammonium group is positively charged at all pH-values. In addition, CNFs contains some amount of carboxyl groups due to residual hemicelluloses. Thus, the c-CNFS molecule has an amphoteric nature. As a result, the net charge of the c-CNFS is pH-dependent (Olszewska et al. 2011a).

2.3.2 Dehydration and hornification

In this section, hornification of cellulose is introduced in order to explain the chemical changes that takes place during dehydration. Cellulose is in swollen state when it is saturated with water. During water removal, the swollen cellulose undergoes dehydration, which leads to gradual structure collapse of the mesoporous swollen state structure. After dehydration, the initial swollen state will not be reached with reimmersion of cellulose in water (Deguchi, Tsujii, and Horikoshi 2008). This phenomenon, which results in irreversible changes in cellulose is called hornification and has been shown to be a result of the absence of hemicellulose and lignin, and is thus observed after hemicellulose and lignin removal (Ding et al. 2019; Langan et al. 2014). Also, nanostructured celluloses, including CNFs are known to be susceptible to hornification (Ding et al. 2019; Klemm et al. 2011).

Ding et al. (Ding et al. 2019) showed that redispersed TEMPO-oxidized CNFs changed significantly in morphology and size after dehydration, aggregated more in radial than axial direction, had decreased transmittance by about 20 percent, and decreased the amount of water content adsorbing on the CNF surface. Introducing of large amounts of substances acting as a steric barrier or containing electrostatic groups can prevent hornification by blocking cooperative hydrogen bonding of the
cellulose chains. Therefore, it has been suggested that CNF hornification could be prevented by the addition of functional groups to CNFs; for example, carboxymethylation has been shown to be very effective in blocking cooperative hydrogen bonding of cellulose (Klemm et al. 2011).

Hornification can be an issue in films or composites that contain CNFs (Klemm et al. 2011), although it could be useful if its role could be better understood in forming new strategies for advanced bottom-up nanotechnologies or assembly of new materials (Ding et al. 2019).

2.3.3 Drying effects

Drying of CNF can be problematic due to the redispersion problems after dehydration, as described in Section 2.3.2. Fiber aggregation may lead to a loss of nanoparticle properties and to sudden shifts from nanoscale material to microscale material. Therefore, the drying process of CNFs requires attention and optimization depending on the purpose and aimed composition after drying.

Peng et al. (Peng, Gardner, and Han 2012) focused on four different approaches of CNF drying: (i) oven drying, (ii) freeze drying, (iii) supercritical drying, and (iv) spray drying. Relevant to the present work, oven drying of CNF suspensions is described in more detail. The water evaporation process during oven drying can be divided into three stages: (i) the constant rate-drying period, (ii) the first falling rate-drying period and (iii) the second falling rate-drying period. (Cichosz and Masek 2019)

The first drying stage involves shrinking of the suspension volume and subsequent CNF movement within the suspension volume. As evaporation occurs on the surface, the water transfer rate from the CNF suspension interior to the surface becomes eventually lower than the water diffusion rate on the surface and the drying moves to the second stage. The drying then takes place inside the suspension and the CNFs begin to gather together, leading to a molecular contact enabled by the impacts of capillary forces and diffusion. The resulting fiber network creates a bulk material as a result of stable, often irreversible intermolecular hydrogen bonding (Cichosz and Masek 2019). The bulk material formation in the oven drying process, reportedly, mainly affected by capillary forces (Peng et al. 2012).
2.4 Alignment of cellulose nanofibers

In their native state, plant-based CNFs are often aligned into highly hierarchical structures, giving plants their optimal mechanical properties. However, despite several attempts and extensive research, realignment of disintegrated and fibrillated CNFs remains challenging, so the superior CNF properties are often challenging to transfer into optimal macroscale performance (Li et al. 2021; Zhu et al. 2020). There are various motivations to align CNFs, such as the need for anisotropic properties; adjusting stiffness and strength of structural materials; optimizing reinforcement efficiency in fiber-reinforced composites; and advancements in biomedical materials and applications (Li et al., 2021), for instance to obtain aligned, ECM-mimicking nanofibrous structures for cell cultivation substrates (Wang, Ding, and Li 2013). Especially relevant to the present work are the need for aligned structures for cell alignment in vitro (Li et al. 2014, 2021), as well as the challenge to fabricate heat-spreading substrates with in-plane anisotropic thermal conductivity (Li et al. 2021; Uetani, Okada, and Oyama 2017); these will be introduced in more detail in Sections 2.5 and 2.6, respectively.

The development of CNF alignment addresses the need of a method to quantify the orientation of CNF and monitor changes in CNF orientation in CFN aerogels, composites and other structures. The most popular method to study CNF orientation is wide angle X-ray scattering (WAXS), which is though limited to crystalline polymers and is not widely available as an expensive instrument. Other methods for investigation of CNF orientation include methods based on Raman spectroscopy, two-dimensional X-ray diffraction, polarized light microscopy, atomic force microscopy (AFM) and scanning electron microscopy (SEM). AFM and SEM are commonly combined with other methods to study CNF orientation. (Ghasemi et al. 2020).

Different cellulose nanomaterials, namely CNCs and CNFs have different properties, and thus remarkable differences in their processability and alignment (Zhu et al. 2020). It is much more challenging to align CNFs compared to CNCs because CNFs are longer and prone to form fiber networks; that is, entangle. Alignment and self-assembly behavior of CNF have received less attention (Li et al. 2021), while CNCs have been extensively reviewed (De France et al. 2020; Habibi et al. 2010; Prathapan et al. 2020) Although extensive research and progress have been made in cellulose nanomaterial alignment, it is still challenging to obtain highly aligned nanocellulose structures in a simple, low-cost and an efficient approach (Zhu et al. 2020).
2.4.1 Methods to align cellulose nanomaterials

Several attempts have been made to align cellulose nanomaterials using a variety of methods, which can be categorized into bottom-up and top-down methodologies. In the bottom-up strategy, the cellulose nanomaterials are tunable before the assembly of the nanoscale molecules into ordered nanoscale materials. In addition, the assembly itself is adjustable and the hierarchical structure at the macroscale can be controlled. The top-down method utilizes the hierarchical structure and already aligned CNFs of plants (Li et al. 2021).

Another categorization aspect of cellulose nanomaterial alignment is the division into stimuli-induced and self-assembly-based methods (Zhu et al. 2020). Because it is relevant to the present work, a short introduction to the available bottom-up methodologies of cellulose nanomaterial alignment, focusing on the alignment of CNFs is provided below.

CNFs have been reported to align using several stimuli-induced methods, such as (i) shear-induced alignment (Zhu et al. 2020), including four subtypes of stretching- or drawing-induced (Josefsson et al. 2015), rotation-shear-induced, coating/casting-induced (Pahimanolis et al. 2013) and flow/extrusion-induced alignment (Håkansson et al. 2014); (ii) electric-field-induced alignment (Kadimi et al. 2014); (iii) magnetic-field-induced alignment (Kim et al. 2008; Kimura and Kimura 2008); (iv) template-assisted alignment (Wicklein et al. 2015); and (v) spinning-induced alignment (Hooshmand et al. 2015). Evaporation-induced self-assembly of CNFs has been reported to a lesser extent (Mariani et al. 2019; Sydney Gladman et al. 2016).

Stimuli-induced techniques often require more time, high energy consumption, a high level of technical expertise, and often expensive proprietary technology than evaporation-induced self-assembly (Mashkour et al. 2013; Zhu et al. 2020). Section 2.4.2 describes the factors that play a role in nanomaterial self-assembly, and Section 2.4.3 focuses on the evaporation-induced self-assembly.

2.4.2 Factors affecting the self-assembly of cellulose nanomaterials

This section focuses on the factors that affect nanomaterial self-assembly in general, and especially those of cellulose nanomaterials. The self-alignment depends on the nanomaterial concentration, charge and length, geometry, colloidal stability of the dispersion, and the evaporation temperature. In addition, the degree of the alignment may be controlled by changing the ionic strength and pH of the solvent (Fall et al. 2008); (iv) template-assisted alignment (Wicklein et al. 2015); and (v) spinning-induced self-assembly (Mashkour et al. 2013; Zhu et al. 2020).
2011, 2013; Zhang et al. 2010; Zhao, Cavallaro, and Lvov 2015). The properties that especially affect self-assembly behavior of CNFs include particle geometry, particle size distribution, surface chemistry, surface charge density, interparticle interactions, and rheological properties (Kontturi et al. 2018; Zhu et al. 2020).

Salas et al. (Salas et al. 2014) described the ability of CNFs to self-assemble at interfaces and highlighted the effect of surface functionalization in controlling these assemblies. For example, surface charge density influences the colloidal stability of CNF dispersion, and together with the surface chemistry they are essential factors in the self-assembly of CNFs. Contrary to charged cellulose nanomaterials, uncharged CNFs tend to entangle and aggregate hindering orientation (Zhu et al. 2020). Besides electrostatic, other interparticle interactions, including van der Waals forces, hydrophobic interaction, and hydrogen bonding have a role in intermolecular interactions during a self-assembly process (Nascimento et al. 2018; Nishiyama 2017). Concentration might play a critical role especially in CNF self-assembly, as CNFs tend to gel at low concentrations (Kontturi et al. 2018).

2.4.3 Evaporation-induced self-assembly of (cellulose) nanomaterials

Nanoparticle droplet drying may result in the formation of assembled structures on the substrate, depending on the mode of solvent evaporation and nanoparticle properties. One of the most common deposited patterns include the “coffee ring” patterns, which resemble the dense ring-like pattern of a dried spilled drop of coffee on a solid surface. However, such ring-like deposits are not characteristic only of coffee, but are seen in a variety of dried drops containing dispersed solutes (Deegan et al. 1997). A coffee ring deposit results when the evaporating boundary line is pinned, and subsequently, the evaporating liquid of the droplet surface is replaced by the outward flowing liquid from the interior, carrying solutes from the interior to the drop periphery. The size of the deposit depends on how long the boundary line was pinned for. In contrast, flow away from the contact line – that is, inward flow may result in a uniform solute deposit (Fischer 2002).

A droplet casting method has been used for the evaporation-induced self-assembly of some polymers, proteins, graphene, and nanoparticles such as carbon nanotubes and metal oxides (Wei and Zhiqun 2012). The evaporation-induced self-assembly of cellulose nanomaterials has been studied by Maskhour et al. (Mashkour et al. 2013) and Parker et al. (Parker et al. 2016). Maskhour et al. (Mashkour et al. 2013) studied CNC alignment and showed their alignment along the evaporating
boundary line. They found that the self-assembly of CNCs is related to the formation of a surface tension torque close to the dry-line boundary layer during evaporation of a CNC suspension. The surface tension torque was considered responsible for the alignment of CNCs. The alignment direction was tangential to the dry-line boundary layer. Surface tension induces a rotating moment that results in orientation of suspended nanoparticles in the proximity of the dry-line boundary. As a result, nanoparticles align parallel to the dry-line boundary. The surface tension gradient and surface evaporation are the two main driving forces close to the dry-line boundary that act on the anisotropic CNCs. This results in surface tension torques proportional to the length of the CNCs and the contact angle between the CNCs and the surface of the substrate, rotating the CNCs and aligning them parallel to the dry-line boundary layer (Mashkour et al. 2013).

The described alignment of CNCs is relevant to the present work. However, although (Li et al. 2021) recently reviewed CNF alignment thoroughly, a review of the literature revealed that there is no report on the self-orientational capacity of CNFs induced by the surface tension torque, evaporation-driven self-assembly, or orientation of charged CNFs in an evaporating droplet meniscus.

2.5 Cell cultivation and cellulose nanofibers

As one of the main application fields of this work is cell cultivation, this chapter briefly introduces the influence of surface properties on cell adhesion and behavior, the potential of CNFs as a cell cultivation substrate, and briefly reviews the studies reporting CNFs in contact with living cells, focusing on TEMPO-oxidized and cationically modified CNFs.

2.5.1 The effect of surface properties on cell adhesion and behavior

The cell-material interaction is a complex process. The most influencing surface properties of a biomaterial are geometry, stiffness and surface charge (Metwally and Stachewicz 2019). Therefore, a lot of effort in tissue engineering is focused on surface modifications and coatings to control wettability, roughness (Brunetti et al. 2010; Dufresne 2013), electrostatic force (Hoshiba, Yoshikawa, and Sakakibara 2018), surface potential (Metwally and Stachewicz 2019), and mechanical properties (Discher, Janmey, and Wang 2005) of the substrate (Metwally and Stachewicz 2019).
The adhesion sites of the cell (focal adhesions) are in the range of 5–200nm. Therefore, they are strongly influenced by nanoscale topography rather than by microscale structures (D. H. Kim et al. 2012). For example, even alterations in roughness of a few nm have been shown to influence cell proliferation (Brunetti et al. 2010). For instance, small variation in fibril size can have an effect on cell proliferation (D. H. Kim et al. 2012).

Cell adhesion to a substrate is essential in tissue engineering. Electrostatic force of the substrate enhances cell adhesion, so charged substrates are widely used for cell culture and to control cell functions (Hoshiba et al. 2018). Surface charge over the surface, as well as chemical composition of the surface, affect many mechanisms in cells. Surface charges determine cells adhesion and later tissue development. Positive charges support regeneration through the activation of signaling cascade of immune system. In addition, the positively charged surfaces adsorb more proteins (Metwally and Stachewicz 2019).

Hoshiba et al. (Hoshiba et al. 2018) demonstrated that cells adhere via both positive and negative charges, emphasizing that the mechanisms of cell adhesion on charged substrates must be reconsidered. The mechanism for how cells adhere to charged substrates remains unclear. In serum-free medium, cells adhere to charged polymers via integrin-independent mechanisms, such as electrostatic and hydrophobic interactions, while in serum-containing medium, biological interaction (integrin-mediated adhesion) plays a significant role in addition to electrostatic and hydrophobic interactions (Hoshiba et al. 2018).

The effects of surface potential on cell behavior are cell-type-dependent because cell behavior is determined by the dominant composition of the ECM. For example, the fibronectin-dominant fibroblast proliferation rate was independent of the degree of negative surface potential, while their morphology changed. Negatively charged fibronectin adsorption on the surface was weak, and fibroblasts altered their morphology to fit the inhomogeneous fibronectin-adsorbed area. For comparison, laminin-dominant epithelial cell morphology was not affected by surface charges, while their adhesion density was enhanced due to positively charged laminin adsorption on the surface (Chang et al. 2016). According to Ribeiro et al. (2020) cells cultivated on charged surfaces have a more elongated morphology. This was dependent on the surface charge, not on the poling direction; that is, whether surface charge was positive or negative. Poling direction had no effect on cell elongation (Ribeiro et al. 2020).

Surfaces with moderate wettability, such as carboxyl group (-COO-) containing surfaces, are found to be beneficial for human fibroblast adhesion and spreading.
(Faucheux et al. 2004). Faucheux et al. (2004) also noticed that the interaction of human fibroblasts with (-CH3) containing group-heads was weak, while strong attachment, spreading and fibronectin matrix formation and growth were observed on carboxyl group-rich surfaces. In addition to wettability, it has been found that increasing surface potential enhances the adhesion and proliferation of human dermal fibroblasts (Altankov, Richau, and Groth 2003).

Cells in native tissues sense and respond to the stiffness of their environment. (Discher et al. 2005) Stiffness of different tissues varies from less than kPa for soft tissues such as fat to more than 10^5 kPa for bone (Skardal et al. 2013). For comparison, the stiffness of the plastic (well plate) is around 10^6 kPa (Skardal et al. 2013). Therefore, the plastic often fails to replicate in vivo elastic moduli. Compared to artificial substrates such as well plate plastics or glass, biological material stiffness values are often closer to those of native tissue types, such as fibrotic tissue (100 kPa), cartilage, and tendon (approaching 10^3 kPa) than common cell culture plastic substrates (Skardal et al. 2013). For instance, the reported stiffness values of CNF films (1–70 kPa) with thickness of approximately 0.1 mm from Kummala et al. (Kummala et al. 2018) resemble those of native tissues such as fat and bone marrow (1 kPa), brain (<2 kPa), lung (<5 kPa), liver (10 kPa), and muscle (10–20 kPa) (Skardal et al. 2013). Therefore, CNF can be considered as a potential material for tissue engineering. Furthermore, it has the advantage of modulating the stiffness by crosslinking (Courtenay, Deneke, et al. 2018).

In contrast to covalent crosslinking in which a permanent structure is formed due to the formation of irreversible chemical links, ionic crosslinking involves the formation of non-permanent network due to reversible link formation. Commonly, ionic bonding is well tolerated and biocompatible due to the lack of crosslinking agent residues (Xu et al. 2018) and improves mechanical properties such as stiffness and elasticity (Lin et al. 2018; Pahimanolis et al. 2013) compared to substrates with covalent crosslinks, which are often less moldable and more brittle (Xu et al. 2018). Pahimanolis et al. (Pahimanolis et al. 2013) created ionic crosslinking between carboxymethyl groups and glycidyltrimethylammonium-chloride-treated CNFs, which increased the dry and wet strength of the composite films.

2.5.2 Cellulose nanofibers in contact with cells

Although plant-derived CNFs have been extensively studied for a wide range of applications, there have been relatively few studies of their interaction with biological
systems, compared to the corresponding studies of the utilization of bacterial nanocellulose. The biocompatibility and/or cytocompatibility of plant-derived CNFs has been demonstrated in many studies (Alexandrescu et al. 2013; Bhattacharya et al. 2012; Ćolić et al. 2015; Hua et al. 2014, 2015a, 2016; Malinen et al. 2014; Wang et al. 2015). Wood-derived unmodified CNF have been shown to have potential especially in 3D cell cultivation (Bhattacharya et al. 2012; Lou et al. 2014; Malinen et al. 2014). In addition, CNFs have been incorporated into wound dressing, partly due to their water-absorbing property (Hakkarainen et al. 2016; Kiiskinen et al. 2019). Wound dressing can also be used as a cell culture scaffold (Kiiskinen et al. 2019) and the surface properties of wound dressings have an effect on cellular behavior. An unmodified CNF surface with a weak negative charge may not be optimal for the growth of mammalian cells (Courtenay et al. 2017). To enhance the cell-surface interactions, the chemical modification of a CNF surface can be beneficial. Adjustment of the hydrophilicity or the surface charge can also be achieved with chemical modifications (Courtenay, Deneke, et al. 2018; Courtenay et al. 2017; Weishaupt et al. 2015).

The surface modification of nanocellulose has been shown to have a direct effect on the cells in contact with nanocellulose substrates. TEMPO-oxidized anionic CNFs have been shown to promote the cell adhesion and growth in many studies (Hua et al. 2014, 2016; Rashad et al. 2017). Human dermal fibroblasts showed a measurable effect on their proliferation and morphology when the effects of changes in nanocellulose surface topography, chemistry, and charge were studied (Hua et al. 2014). Hua et al. (Hua et al. 2015a) showed an improved cytocompatibility of EPTMAC-modified cationic CNFs. In addition, cationic CNFs were reported to facilitate cell attachment through electrostatic interactions between the phosphate-lipid bilayer of the cell membrane and the positively charged quaternary ammonium group in the absence of matrix ligands; that is, serum-free cell cultivation medium (Courtenay et al. 2017). Hua et al. (Hua et al. 2016) suggested that substrate CNF fiber alignment could have an advantageous effect on cell adhesion and spreading. They observed fiber alignment with anionically charged CNF films that had been TEMPO-oxidized, prepared by vacuum filtration. They controlled the number of negatively charged groups and found that increased surface charge resulted in increased unidirectional fiber alignment. (Hua et al. 2016) In addition, He et al. (He et al. 2014) observed cellular alignment on an aligned nanofiber scaffold fabricated with electrospinning.
2.5.3 Anisotropic structures in biomedical sciences

An anisotropic architecture in most native ECMs of tissues or organs is important for tissue function (Liu, Thomopoulos, and Xia 2012; Wang et al. 2013). Therefore, organized structure that resembles the topographical structures provided by fibrous components of ECMs is believed to be crucial for guiding cell growth or tissue regeneration (T. G. Kim, Shin, and Lim 2012; Wang et al. 2013; Yang et al. 2005). At the cellular and molecular level, cell alignment also plays an important role in cytoskeleton reorganization, membrane protein relocation, nucleus gene expression, and ECM remodeling. In addition, cell alignment helps modulate the mechanical properties of such tissues as skeleton, cardiac muscle and tendon. (Li et al. 2014) Cells are commonly cultured in vitro on a variety of substrates. The morphology of the cell cultivation substrate plays a key role in cell adhesion and cell elongation, as cells commonly adjust their morphology according to the underlying substrate (H. N. Kim et al. 2012). Therefore, there is a need to engineer substrates with features that support cell alignment in vitro in order to study and develop new solutions in the area of biomechanics, cell biology, tissue engineering and regenerative medicine (Li et al. 2014).


Surface topographical features can also be used, for instance, in enhanced repair process of neural damages, since they act as cell guidance structures leading to improved cellular differentiation and axonal reconstruction (Ghorbani and Zamanian 2016; Malkoc et al. 2015). A single aligned cell layer can also initiate the development of an organized structure of cell layers and tissues, such as cornea, vascular media, and dermis (Guillemette et al. 2009).

Methods to produce such aligning channels, grooves, and surfaces include deep reactive ion etching (Anene-Nzelu et al. 2013), electron beam lithography (van Delft et al. 2008; Gamboa et al. 2013), direct laser writing (Zhou et al. 2012), using a
femtosecond laser (Hribar et al. 2015), photolithography (He, Halberstadt, and Gonsalves 2004), plasma dry-etching (Lu et al. 2008), dual etching and bioprinting (Bhuthalingam et al. 2015), and electrospinning (Wang et al. 2013). Electrospinning can be used to align fibrous structures that are of significant interest for tissue-engineering purposes (Cai et al. 2012; Chen and Su 2011; Jose et al. 2009; Wang et al. 2013; Xie et al. 2010).

Aligned nanofibrous structures have shown superior capacity in shaping cell morphology, guiding cell migration, and affecting cell differentiation when compared to other types of structures both in vitro and in vivo (Chen et al. 2012; Huang et al. 2012; Wang et al. 2013; Xie et al. 2010). Cells, their cytoskeletons, and their nuclei have been shown to align and elongate parallel to the fiber axes, when cultured on aligned fibers (Chew et al. 2008). The challenge is to create an aligned porous 3D structure of fibers with a diameter identical to that of native ECM fibers (a diameter less than 100 nm, preferably in the range of 10–50 nm) (Holzwarth and Ma 2011; Liu et al. 2012; Wang and van Blitterswijk 2010; Wang et al. 2013). A technically effortless and energy-efficient method for producing repeatable aligned substrate layers for the initiation of organized structure development is needed (Guillemette et al. 2009; Mashkour et al. 2011; Sundar, Sain, and Oksman 2010).

2.6 Hybrid nanocellulose/nanocarbon composites

There is increasing demand for thinner, cheaper, lighter, and more eco-friendly electrically conductive films and composites. (H. Zhang et al. 2019). CNF nanocomposites can be classified into 1D structures (including fibers), 2D structures (such as films and coatings), and 3D structures (such as molded, extruded, and 3D-printed structures) (Clarkson et al. 2020) and hydrogels (Nascimento et al. 2018), respectively. The present thesis focuses on composite films, and 3D structures are out of the scope of this study. The techniques for fabrication of CNF-based nanocomposites include solvent casting, melt mixing, in situ polymerization, and electrospinning.

The hydrophilic character of CNF and its tendency to form strong networks held through hydrogen-bonding hamper its uniform dispersion within hydrophobic matrices. Therefore, surface modification is often required in order to improve compatibility and homogeneous dispersion within hydrophobic matrices (Kalia et al. 2014). Incorporation of hydrophilic CNF in non-polar matrices is challenging as it often results in poor adhesion between components, which leads to weak interfacial
bonding. Therefore, CNF is commonly dispersed into other materials using an aqueous dispersion (Salas et al. 2014). CNFs can be combined with 2D nanomaterials to produce, for example, nanocellulose films with enhanced performance, novel functions, or both. CNF is not only a host matrix for other nanomaterials, but the aim is to take advantage of both nanomaterial properties and create novel properties for the composite. CNF often acts as a green dispersant to uniformly disperse or stabilize other nanomaterials. Compared to plain CNF films, CNF-nanoparticle films can have enhanced mechanical, optical, and barrier properties and/or novel functionalities (Fang et al. 2019). Relevant to this work, electrically conductive CNF composite films are introduced in more detail.

2.6.1 Electrically conductive cellulose nanofiber-containing composite films and hydrogels

A common way to produce electrically conducting CNF-based materials has been coating, blending, or doping CNF with conductive nanoparticles such as conductive polymers, graphene, inorganic nanoparticles, ionic liquids, or carbon nanotubes. CNF is often used to add biocompatibility of the resulting composite, although this sets some requirements for the arrangement of nanoparticles. For such purpose, the conducting component is introduced into the CNF matrix. In addition to biocompatibility, CNFs have other beneficial properties. For instance, they have film- and hydrogel-forming potential and can be a stable and robust carrier, matrix, or scaffold component for the fabrication of new functional materials (Shi, Phillips, and Yang 2013). There is growing demand for thinner, cheaper, lighter, and more eco-friendly electrically conductive composite films. A CNF, as a primary ingredient in such composites, can be considered an eco-friendly and low-cost substrate option (H. Zhang et al. 2019).

CNF-based electrically conductive composites have many advantages, although, many challenges remain related to electrical connectivity, tolerance to forces and extension, interfacial adhesion, and chemical stability. It is the mobility of the electrons that determine the conduction within the composite material. Electrically conductive composites generally contain both well-conducting and insulating phases (Figure 2), which inevitably results in the presence of gaps, defects, or a complete lack of contact among the electrically conductive phase of the composite (H. Zhang et al. 2019). Relevant to this work, multiwalled carbon nanotube e-CNF composites are introduced in more detail in the following section.
2.6.2 Carbon nanotube nanocellulose composites and applications

Carbon nanotubes (CNTs) are categorized into single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), which consist either of a single layer of graphene sheet rolled into a cylindrical tube or of multiple concentric graphene layers, respectively (Shi et al. 2013). The morphology of CNTs resemble that of CNFs (Bacakova et al. 2020). CNTs have exceptional mechanical, electronic, thermal, optical, and magnetic properties, which make CNTs excellent candidates to act, for example, as reinforcing agents and/or electrically conductive components in CNF composites (Shaobo Zhang et al. 2018) for applications in both physical and life sciences (Shi et al. 2013).

Despite their potential as functional nanocomposites, combining and processing of CNFs and CNTs presents challenges because CNF and CNTs are both insoluble in water and common organic solvents (Qi et al. 2015). In addition, CNTs have a tendency to self-aggregate due to van der Waals and hydrophobic interactions between their sidewalls, which has hindered their application (Shaobo Zhang et al. 2018). However, chemical modifications of at least one of the nanomaterial components may alter the situation, as the presence of repulsive forces between nanocomponents can be expected to have a dispersing effect. Therefore, depending on the process by which the composite is formed, the conductive particles may be kept from agglomerating and contacting each other within the resulting composite (H. Zhang et al. 2019). For example, Koga et al. (2013) showed that TEMPO-
oxidation of the CNF has enhanced the dispersion of CNTs when mixing these two nanomaterial components.

The addition of functional nanomaterials, including CNTs, during the assembly of nanocellulose, endows the resulting film with multifunctionalities such as electrical and thermal conductivity (Fang et al. 2019). Anisotropic properties are occasionally needed in advanced materials. However, it is extremely challenging to align CNF in composites (Li et al. 2021). Thanks to their amphiphilic nature, CNFs can effectively disperse CNTs in water prior to the fabrication of nanocellulose film. The dispersion effect can be enhanced with CNF modifications in which CNFs end up with a higher charge density than native CNFs (Fang et al. 2019; Hajian et al. 2017).

Materials with anisotropic thermal conductivity have a potential use as heating-guiding materials with both heat-insulating and heat-diffusing areas that could help to control the heat flow in future thin electronics (Zhu et al. 2020) and in preventing local failures caused by accumulated thermal energy as well as in applications in which the control of heat flow between directions is desired (T. Li et al. 2018). As the thermal conductivity of CNFs is extremely low, they are generally considered good insulating material (T. Li et al. 2018). Li et al. (T. Li et al. 2018) prepared an anisotropic, thermally insulating bulk material with aligned CNFs. Alignment of CNF resulted in anisotropic thermal properties, with two-fold thermal conductivity along the cellulose alignment directions compared to the transverse directions; that is, perpendicular to the CNF alignment. Lavoine and Bergström (Lavoine and Bergström 2017; Uetani et al. 2017) also showed that aligned cellulose nanomaterials provide anisotropic thermal conductivity. Despite the previous work, the development of heat-spreading substrates with in-plane anisotropic thermal conductivity remains challenging (Li et al. 2021).

As bulk alignment of CNF-based materials can be challenging, it is significantly more difficult to align fibers in polymer composites (Li et al. 2021). According to Li et al. (Li et al. 2021) the orientation of CNFs in polymer materials is scientifically and technically challenging, although a few studies have reported on composites with aligned CNFs (Mohammadi et al. 2019; Nissilä, Hietala, and Oksman 2019; Oksman, Mathew, and Sain 2009). The major obstacle regarding the wider use of CNFs in composites are the compatibility issues with polymer matrix, excess water carried in CNFs, and poor interfacial adhesion (Clarkson et al. 2020).
2.7 Summary of the current challenges

Despite several attempts and extensive research, realignment of disintegrated and fibrillated CNF remains challenging, so the superior CNF properties are often challenging to transfer into optimal macroscale performance (Li et al. 2021; Zhu et al. 2020). It is challenging to obtain highly aligned nanocellulose structures in a simple, low-cost, and efficient manner (Zhu et al. 2020). For instance, stimuli-induced techniques require time, high energy consumption, a high level of technical expertise, and often expensive technology. Thus, there is a demand for simple, energy efficient methods (Mashkour et al. 2013; Zhu et al. 2020).

Another aspect of challenges is related to combining CNFs with MWCNTs. Such a combination has potential as functional nanocomposites, but also involves challenges because CNFs and MWCNTs are both insoluble in water and common organic solvents (Qi et al. 2015). In addition, MWCNTs have a tendency to self-aggregate due to van der Waals and hydrophobic interactions between their sidewalls, which has hindered their application (Shaobo Zhang et al. 2018). Furthermore, alignment of CNFs is particularly challenging in the presence of other components, which hinders its potential use in composite film applications in which the properties of aligned CNF would be favored. For instance, there is increasing demand for thinner, cheaper, lighter, and more eco-friendly electrically conductive films and composites.
The main aim of this dissertation was to develop self-assembly methods for creating aligned and functional cellulose nanofiber and composite film surfaces. This dissertation is comprised of three publications: Studies I–III (Figure 3). The aim of Study I was to develop surfaces with aligned CNF and study cell growth and orientation on the aligned surfaces. The objective of Study II was to improve the cytocompatibility of a low-cost cellulose mesh using charged CNF coatings and compare cell behavior on anionic and cationic CNF coatings. The aim of Study III was to develop a method to align c-CNF in presence of MWCNT component to obtain electrically anisotropic nanocomposite films. The specific aims of this dissertation are:

1. To develop methods for producing anisotropic cellulose nanofiber (CNF) and CNF/CNT-composite surfaces (Studies I and III)
2. To develop CNF substrates suitable for cell growth (Studies I and II) and improve cytocompatibility of commercial cellulose mesh using CNF coating (Study II)
3. To develop a method for guiding cell orientation on surfaces with aligned CNFs (Study I)
4. To investigate ways to control cell growth or nanocomposite film properties using charged CNFs and self-assembly. (Study I, II and III)
5. To develop a method for producing a free-standing film with in-plane anisotropic thermal and electrical conductivity (Study III)
AIMS OF THIS STUDY

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Figure 3. Summary of publications Study I, Study II and Study III, and used nanomaterials. Connection between studies is illustrated as overlaps of the squared areas of each study.
4 MATERIALS AND METHODS

4.1 Cellulose nanomaterial preparation

CNFs used in Studies I–III were obtained from VTT, the Technical Research Center of Finland. Bleached and never-dried cellulose kraft pulps were used for the production of CNFs. Three different CNF grades were used in Studies I–III: native CNF (u-CNF) in Study I, anionic CNF (a-CNF) in Studies I and II, and cationic cellulose nanofibers (c-CNF) in Studies I–III.

The native grade (u-CNF) was produced from hardwood pulp, which was converted to the sodium form prior to fibrillation (Lahtinen et al. 2014). Subsequent to ion exchange, the pulp was soaked at 1.7 percent consistency and dispersed using a high-shear Ystral X50/10 Dispermix mixer for ten minutes at 2000 rpm. The resulting pulp suspension was prerefined in a grinder (Supermasscolloider MKZ A10-15J, Masuko Sangyo Co., Japan) at 1500 rpm, and supplied through chambers with diameters of 400 µm and 200 µm. The following nine passes were supplied through 400 µm and 100 µm chambers. After 10 passes, the gel was produced using the operating pressure 1800 bar. After the mechanical treatment, the fiber slurry was homogenous and viscous gel with a final solid content of 1.5 percent.

Two different production batches of anionic grade (a-CNF) were used in Studies I and II. Both were produced from softwood kraft pulp using TEMPO-mediated oxidation. TEMPO and NaBr were used as catalysts in oxidation reaction with NaOCl. The pH was maintained at 10.5 (Study I) or 10.3 (Study II) with the addition of 1 M NaOH during the reaction. After stabilization of the pH, the reaction was stopped with the addition of ethanol into the oxidized pulp suspension. The pH was finally adjusted to 7 (Study I) or 6.5 (Study II) by adding HCl. The oxidized pulp was washed with deionized water by filtration and stored in a fridge prior to mechanical fibrillation. The carboxyl content of the oxidized pulp was determined using conductometric titration according to the method described by Saito et al. (Saito et al. 2006). In the mechanical fibrillation used in Study I, the oxidized pulp was soaked at 1.5 percent solids and dispersed using a high-shear Ystral X50/10 Dispermix mixer for 10 minutes at 2000 rpm. Subsequently, the pulp suspension was fed into a Microfluidics microfluidizer type M110-EH at 1800 bar. The suspension twice
passed the chambers with diameters 400 µm and 100 µm. After the first pass, the gel was diluted to 1 percent consistency and supplied into the microfluidizer for the second pass. As a result, a viscous and transparent hydrogel was obtained with a final dry material content of 1.16 percent and a charge value of 1 mmol/g dry pulp. In Study II, the mechanical fibrillation process was similar to Study I and is described in more details in the corresponding publication. The final dry material content in Study II was 1.45 percent.

The cationic grade (c-CNF) was produced from hardwood pulp and the following procedure was used in all studies. Cationization was conducted in a method similarly to that of Bendoraitiene et al. (Bendoraitiene et al. 2006), who reported the cationization of starch using EPTMAC (Raisacat, Chemigate) as a cationizing agent. The pulp was first concentrated in an oven to 63 percent dry matter content. The reaction mixture was prepared from 140 ml of EPTMAC, 2 g of aqueous solution of NaOH 5 percent, and 2.3 ml of water. The ingredients were mixed and 50 ml of water was added to the mixture, which was warmed to 45°C. The pulp was added to the mixture and stirred for 24 h at a high cellulose consistency. After the reaction, the cationic pulp was washed with ethanol, tetrahydrofuran, and water. Functionalized cellulose pulp was diluted to 2 percent concentration and dispersed using a high-shear Ystral X50/10 Dispermix for 10 minutes at 2000 rpm. The pulp was then fibrillated using a Microfluidics microfluidizer type M110-EH at 1800 bar pressure. The suspension was inserted twice through two chambers with diameters 400 µm and 100 µm, respectively. The fibrillated c-CNF formed a highly viscous and transparent hydrogel with a final dry material content of 2.01 percent. The degree of substitution was analyzed according to the method proposed by Bendoraitiene et al. (Bendoraitiene et al. 2006) and was 0.35.

The CNF hydrogels were further pretreated to obtain homogenous suspensions in comparable and optimal concentration for further use. In Studies I, II and III, the CNF gels were diluted to 0.15 percent (w/v) suspensions in Milli-Q water and sonicated for two minutes at 20 percent amplitude using a SONICS Vibra Cell VCX 750 ultrasonic processor (Sonics and Materials, Inc., USA). To remove larger fibers, the sonicated samples were centrifuged at 10,000g for 60 min (Thermo Scientific SL 8). The resulting supernatant was used for the preparation of surfaces on glass cover slips in Study I (Section 4.2.1), coatings on cellulose meshes in Study II (Section 4.2.2) and multicomponent systems used to prepare nanocomposite films on PDMS substrate in Study III (Section 4.2.3).
4.2 Preparation of cellulose nanofiber and cellulose nanofiber/carbon nanotube-composite substrates

4.2.1 Cellulose nanofiber surfaces

CNF supernatants described in Section 4.1 were used to prepare CNF surfaces (u-CNF, a-CNF and c-CNF) investigated in Study I. Glass cover slips were used as a substrate for the CNF surfaces. To achieve better adhesion between the CNFs and the glass, polyvinylamine (PVAm) coatings were prepared by dispensing 300 µL of 1 mg/mL polymer solution on the glass cover slips, followed by a 15 min incubation at room temperature and washing with deionized water. Volumes of 2 µL or 200 µL of CNF-supernatants were droplet casted on PVAm-coated surfaces. In addition, the c-CNF supernatant was used to “print” lines on surface in a process similar to contact printing. Surfaces were dried in an oven at 60°C or at varying temperatures (37–60°C) on top of an ITO heat plate (H401-Glass-K-Frame, OkoLab) compatible with an optical microscope.

4.2.2 Cellulose nanofiber coatings

Cellulose mesh used in Study II as a substrate was highly pure spunlace nonwoven cotton fabric PurCotton® (Winner Industrial Park, Shenzhen, China), which was cut into the 1.5 x 1.5 cm² samples (“non-coated meshes”). In order to prevent any movement or floating of the meshes during cell cultivation, the meshes were fixed prior to coating into the CellCrown™ inserts (Scaffdex Ltd., Tampere, Finland) that were fitted into the 24-well cell culture plates (TPP, Trasadingen, Switzerland).

Different volumes of CNF supernatant described in Section 4.1 were used to prepare the CNF coatings (a-CNF and c-CNF) on cellulose meshes, investigated in Study II. Either 150 µL or 600 µL volumes were used to coat the meshes with either a-CNF or c-CNF, while 300 µL of c-CNF and 300 µL of a-CNF supernatants were used for the combination coating. The resulting sample types are referred to as c150, c600, a150, a600, and c+a, respectively. A pristine coated cellulose mesh was used as a control. The CNF-coated samples were dried for 24 h in a laboratory dryer (Binder, Tuttingen, Germany) at 50 °C.
4.2.3 Nanocomposite films

Films used in Study III are nanocomposite films, either isotropic or anisotropic and their preparation is described. In order to prepare the nanocomposite films, two-component dispersion and three-component suspensions were first produced, as described below. Isotropic control films were fabricated from high-energy-sonicated (hes-)c-CNF-MWCNT two-component dispersion. Anisotropic films were fabricated from three-component suspensions containing two-component dispersion and additional c-CNF.

Multiwall carbon nanotubes (MWCNT, Nanocyl 7000, Nanocyl SA., Sambreville, Belgium) used in Study III were purchased from Nanocyl Inc. and the product was used in the state it was in when received. MWCNT and c-CNF were used to process the dispersions in Study III. The c-CNF supernatant with 0.145 w% was used for c-CNF-MWCNT dispersion. The dry w% of the supernatant was determined from the freeze-dried product.

In Study III, the isotropic control films were prepared using a two-component dispersion. The film production is similar between isotropic and anisotropic nanocomposite film – only the preparation of two-component dispersion and three-component suspension differs. The control hes-c-CNF-MWCNT dispersion contained c-CNF supernatant with a concentration of 0.15 w% of and MWCNT in aqueous medium. The dispersion was prepared by sonicating c-CNF supernatant and MWCNTs in 100 mL glass beakers to obtain a homogeneous stable dispersion. In sonication, a tip horn sonicator Q700 (QSonica LLC, Newton, CT, USA) was used using a constant amplitude. Samples were sonicated using constant energy per dry mass, respectively 625 kJ/g. The sonication energy optimized in previous studies of Siljander et al. (Siljander et al. 2018, 2019) were selected. The total dry mass of the control sample was 0.16 g. The resulting hes-c-CNF-MWCNT dispersion was used to prepare control nanocomposite films.

Anisotropic films were fabricated using hes-c-CNF-MWCNT dispersion and additional c-CNF, which was mixed with the dispersion, resulting in a three-component suspension. Three different anisotropic films were manufactured using three different three-component suspensions. These three different samples are later referred to as Sample 1 (S1), Sample 2 (S2) and Sample 3 (S3). Additional c-CNF in three-component suspension of S1 and S2 was the c-CNF supernatant described in Section 4.1. In three-component suspension S3, the c-CNF supernatant described in Section 4.1 was further sonicated (625 kJ/g) before mixing with hes-c-CNF-MWCNT dispersion. The hes-c-CNF-MWCNT dispersion was suspended with
varying volume ratios of additional c-CNF for the preparation of three-component suspensions S1 (37.5 percent v/v), S2 (20 percent v/v) and S3 (20 percent v/v). Thus, S1 and S2 differ in the volume ratio of the dispersion and the added c-CNF, while S2 and S3 differ in the pretreatment of the added c-CNF, while the volume ratio is kept constant. As a result of mixing the hes-c-CNF-MWCNT dispersion with c-CNF, a three-component suspension was formed and used for anisotropic nanocomposite film production in Study III.

The film production described here is similar between isotropic and anisotropic nanocomposite film; only the preparation of two-component dispersion and three-component suspension differs. To fabricate nanocomposite films, polydimethylsiloxane (PDMS) substrates were fabricated with standard PDMS curing procedure. A PDMS layer approximately 3 mm thick was cut into 10 mm diameter circular substrates using a 10 mm punch. The nanocomposite films were prepared by casting 250 µL of the dispersion (control sample C) or suspension (samples S1, S2, S3) on PDMS substrates and drying 5 h at 60 °C to obtain self-standing circular nanocomposite films with 10 mm diameter and thickness between 7 µm and 11 µm.

The two-component dispersion produces control nanocomposite isotropic control films, while three-component suspensions S1, S2, and S3 produce corresponding assembled anisotropic nanocomposite films. Thus, all films in Study III are nanocomposite films, either isotropic or anisotropic.

4.3 Characterization of the cellulose nanofiber materials, substrates and their performance

This Section introduces the characterization of the CNF-based substrates prepared in Studies I, II, and III. These substrates include CNF surfaces in Study I, CNF coatings in Study II, and c-CNF/MWCNT nanocomposite films in Study III. The details of the characterization methods used can be found from original publications (Studies I–III).

4.3.1 Contact angle measurements and droplet evaporation dynamics

Wetting of the CNF surfaces was analyzed in Studies I and II using an OCA-15 plus optical goniometer (Dataphysics Instruments, GmbH, Germany). A 2 µL drop of
dH₂O (Study I and II) or Dulbecco’s modified Eagle medium (DMEM) (Study II) was dispensed on the CNF surfaces, and the resulting side profile photograph of the droplet was captured with the goniometer at 0.2-second intervals to determine the static contact angle.

Droplet evaporation dynamics of c-CNf suspension were investigated in Study I in order to explain c-CNf alignment along the evaporating droplet boundary line. To investigate the evaporation dynamics using image-based analysis, droplet evaporation was recorded. Evaporation was examined using an inverted microscope (Zeiss AxioObserver.Z1, Germany) and an OCA-15 plus goniometer in order to obtain detailed information on the evaporation. The side profile images were captured using a frame interval 1 s in the contact angle measurement setup to enable a detailed analysis of the contact angle and the contact line behavior during evaporation. The contact angle and the width of the droplet base (mm) as a function of time was determined from the captured images. To observe the evaporation dynamics, droplet evaporation was recorded under the optical microscope using frame intervals between 11 ms and 51 ms. A droplet was cast on a cover slip on a heat plate (at 60°C) and each experimental setup (c-CNf droplet evaporation on PVAm coating, and dH₂O droplet evaporation on glass and PVAm) was repeated at least three times. Image-based analysis was conducted on the recorded data to show the displacement of the contact line as a function of time (during evaporation). The propagation of the droplet edges towards the center was tracked and plotted as a function of time. A dark droplet was clearly discernible from its light background, and a binary image of each frame was formed by appropriate thresholding. The edge was located from each binary image with 0.5 pixel accuracy. The pixel size was 1.17 μm. The location of the center of the droplet was determined from the last frame in which the droplet was present before it vanished.

4.3.2 Swelling ratio and water absorption

Water absorption, referred to as the swelling ratio (SR) of noncoated meshes, coated a600, c600, c+a meshes, and the corresponding CNF-coated glass coverslips, were measured in Study II in order to explain the material behavior in liquid and cell responses on different samples during time. The swelling ratio was calculated from the weight of the samples according to Equation 1, where \( W_0 \) is the initial weight and \( W_s \) is the weight of the swollen sample at measurement time point.
\[ SR = \frac{w_s - w_o}{w_o} \] (1)

The swelling ratios of the meshes and CNF-coatings at 37°C were studied at Time Point I (20 min) and Time Point II (17–20h) in cell culture medium (DME), and in deionized water (dH2O).

### 4.3.3 Electron microscopies

CNF surfaces (Study I), CNF coatings (Study II) and nanocomposite films (Study III) or the raw materials used in their fabrication were examined under helium ion microscopy (HIM; Orion NanoFab, Zeiss, Germany) was conducted. To visualize the surface topography and the nanomaterial components of the nanocomposite films in Study III, scanning electron microscopy (SEM) and (scanning) transmission electron microscopy ((S)TEM) were used. Images of the nanocomposite films were scanned using SEMs (UltraPlus, Carl Zeiss, Oberkochen, Germany; JSM-IT500, Jeol Ltd., Tokyo, Japan). The samples were mounted to aluminum SEM stubs using carbon tape or carbon glue. The surface of the nanocomposite films, and the teared film edges were imaged to observe the cross-sections of the films. Either carbon or gold-coating was used to avoid charging of the sample during the SEM studies. (S)TEM (JEM-F200, Jeol Ltd., Tokyo, Japan) was used to characterize untreated MWCNTs and small pieces of teared nanocomposite film edges fixed on TEM grids with a holey carbon film.

### 4.3.4 Characterization of mechanical properties and surface roughness using atomic force microscopy

Surface roughness of the CNF surfaces and the CNF coatings were measured in Studies I and II, respectively. Atomic Force Microscopy (AFM) was used to observe fiber arrangement on the droplet evaporated CNF surfaces (Study I) and nanocomposite films (Study III). AFM (XE-100, Park Systems, USA) scanning was performed in a tapping mode using a standard ACTA AFM probe (AFM probe, USA) with a resolution of 256x256 pixels. The size of scanned area was 1x1 μm² or
5x5 μm². Images were analyzed using Matlab R2016a (The MathWorks, Inc., USA), and CytoSpectre (Kartasalo et al. 2015); see Section 4.6 for more details.

To investigate the fiber arrangement in Study I, an arithmetic average height parameter (Rₐ) was obtained from several points (n=27) of the AFM images. In addition, surface roughness was analyzed with Matlab from AFM scans separately in horizontal (rows) and vertical (columns) directions (n=256 in each direction). A spacing parameter called high spot count (HSC), described in (Gadelmawla et al. 2002), was obtained from the horizontal repetition over aligned and more random surfaces. In the HSC determination, the “selected level” parameter (Gadelmawla et al. 2002) as 10 percent of the height of the highest peak above the zero average line was used. The “average wavelength” (λₛ) parameter (Gadelmawla et al. 2002) was obtained to estimate the spacing and the length scale of the repetitive aligned structure. The fiber alignment was quantified using the spectral analysis of orientation method provided by the CytoSpectre software.

In Study II, AFM data was acquired for the samples coated with c600, a600, and c+a. The coatings were prepared by a gradual application of 600 μL of CNF suspension on both sides of the cellulose mesh. Young’s modulus and the Rₐ were determined on the coating from the outer side of the samples using an Olympus IX 81 camera (Japan) linked with a JPK NanoWizard 3 AFM microscope (JPK, Berlin, Germany). Roughness maps of the dry samples were gathered using an SNL-10A probe (Bruker AFM Probes, Billerica, MA). The size of each scanned area was 10x10 μm² with a resolution of 128x128 pixels. The dry samples were further examined for their mechanical properties in the same setup as the roughness mapping but with an MPP-121210-10 probe (Bruker AFM Probes, Billerica, MA). The force-distance curves of dry samples were acquired by using force spectroscopy mapping and scanning the samples at the grid size of 25x25 μm² with resolution of 8x8 pixels.

The mechanical properties of the moist samples were studied at time points of 20 min and 17 h after immersion in DMEM using a colloidal probe (NanoAndMore, Wetzlar, Germany). The absolute value of Young’s modulus was determined. Two parallel samples (eight measurements in total) for each experimental group were used, and the results were presented as the median and the interquartile range.

4.3.5 Chemical characterization of the nanomaterials using NMR and FTIR

In Study III, sonication-induced chemical alterations between c-CNF samples were investigated using nuclear magnetic resonance (NMR) spectroscopy and fourier
transform infrared (FTIR) spectroscopy. NMR was also used to characterize the interaction between c-CNf and MWCNT after sonication (Study III).

The following samples were prepared and freeze-dried for NMR studies. Cationic CNf hydrogel (2.01 w%) was diluted to 0.15 w% (W/v) concentration in D2O (Sigma Aldrich), referred to as Sample 1 (untreated c-CNf). Samples 2–3 were sonicated and centrifuged as described in Section 4.1. To study the effect of sonication, the resulting supernatant was sonicated using 625 kJ/g (Samples 2 and 3) described in Section 4.2.3. All samples were freeze-dried before NMR experiments to remove excess moisture.

NMR spectra were measured using 500 MHz JEOL JNM-ECZ 500R spectrometer. Samples (approx. 50 mg) were packed into a 3.2 mm diameter zirconia rotors with KelF caps as a tick suspension in D2O. The semi-solidstate FG-MAS 1H spectra were measured at room temperature, with the high-resolution field gradient FG-MAS probe at a spinning rate of 5 kHz spinning rate. A water suppression pulse sequence was applied during the measurements.

FTIR spectra (Bruker Tensor 27 FTIR spectrophotometer, Billerica, USA) were measured using Attenuated Total Reflection (ATR-)FTIR Diamond crystal. Samples 1–3 described above were measured before and after sonication for comparison of the absorption bands of the untreated and sonicated samples.

4.3.6 Micro-CT characterization of the meshes and nanocomposite films

Uncoated and c-CNf -coated mesh samples in Study II and nanocomposite films in Study III were characterized using X-ray microtomography (micro-CT) at MicroXCT-400 (X-ray tube voltage of 40 kV and a current of 250 µA; Carl Zeiss X-ray Microscopy, Inc., Pleasanton, CA, USA). Avizo 2019.3 software (Thermo Fisher Scientific, Waltham, MA) was used for the data visualization.

In Study II, the noncoated meshes were imaged with micro-CT. The 3D micro-CT images were reconstructed from 1601 projections with a four-second exposure time (20× objective, binning 2, pixel size 1.15 µm) using the micro-CT software tool XMReconstructor. 3D image stacks were manually thresholded for the 3D analysis. The mean fiber thickness and the mean void thickness with standard deviations, volume fraction, and porosity were calculated by BoneJ Fiji plugin (Doube et al. 2010; Palmroth et al. 2020; Schindelin et al. 2012).

In Study III, micro-CT was used to determine the thickness of the nanocomposite films. The 3D micro-CT images were reconstructed as described.
above, with a 10-second exposure time (pixel size 1.048 µm). The mean film thicknesses, with standard deviations from different locations of the films, were calculated with the BoneJ Fiji plugin.

4.3.7 Electrical characterization of the nanocomposite films

In Study III, differences in electrical properties of the assembled and control nanocomposite films were studied using infrared imaging and resistance measurements.

Infrared imaging (IR-imaging, Fluke Ti400, Everett, WA, USA) was used to investigate the electrical anisotropy of the assembled c-CNF+hes-c-CNF/MWCNT films and uniform conductivity of the control hes-c-CNF/MWCNT nanocomposite films. Silver ink contacts were applied to the opposite edges of the film samples. Current was inserted through the silver contacts and film heating was recorded.

 Resistances of nanocomposite films were measured according to the measurement plan presented in Figure 8 of Study III. The resistance was investigated along (i) the center line through the sample (Measurements A–C), and (ii) along imaginary Circles 1 and 3 (Measurements D and E, respectively). The resistance between each measurement point was calculated from current outputs measured with a constant voltage 0.5 V using a two-terminal measurement with a potentiostat (Iviumstat, Ivium Technologies B.V., Eindhoven, The Netherlands). An average of current signal measured for 10 s from each measurement point was used in the resistance calculation \( R = U / I_{\text{avg}} \). Three parallel samples of each nanocomposite film type (S1, S2, S3 and control) were used with 3–5 repeated measurements for each film in order to show the repeatability of the presented assembly. Matlab was used for data analysis. The results were presented as the average resistance of the parallel films, and the repeat measurements \( (n=9) \) were obtained with a 95 percent confidence interval.

To ensure the repeatability of measuring locations, a measurement mask with circular openings (Figure 8b of Study III) was used. The measurement locations were on the center line in the radial direction, referred to as a radial line (locations from 1 to 9), as well as on Circles 1 and 3 (locations 1, 45°11, 45°12, 90°11, 90°12, 135°11, 135°12, 180°1 and 3, 45°31, 45°32, 90°31, 90°32, 135°31, 135°32, and 180°3, respectively). In the notation used in Measurements A–C (Figure 8 in Study III), \( n_\text{a} \)–\( p_\text{n} \) represent the locations of the negative \( (n_\text{a}) \) and positive \( (p_\text{n}) \) probe, respectively, where \( a \) refers to the location of either probe on the film.
During Measurements A and B (Figure 8 in Study III), the locations of both probes were changed. In Measurements C, D, and E, the negative probe remained unchanged either in Location 1 (Measurements C and D) or in Location 3 (Measurement E), while the location of the positive probe varied. In the notation used in Measurements D and E, $\alpha_{yn}$ represent the locations of the positive probe, where $\alpha$ describes the angle between the lines formed along the location of the positive probe and the center and along the location of the negative probe and the center, while $y$ represents Circle 1 or Circle 3 and $n$ represents the location of the probe on the film on either one of the halves ($1$ for the upper half and $2$ for the lower half). Measurement locations $180^\circ_1$ and $180^\circ_3$ correspond to the Locations 9 and 7 at the radial line, respectively. The distance between the negative and the positive probe is equal in the Locations $1-45^\circ_{11}$ and $1-45^\circ_{12}$ and are therefore paired in the result chart.

4.4 Cell culture

In this dissertation, cell behavior on CNF surfaces and coatings was studied, using mouse embryonal fibroblasts (MEF) in Study I, as well as adipose-derived stem cells (ADSC) and neonatal normal human dermal fibroblast (NHDF) in Study II. All the surfaces intended for cell culture were exposed to ultraviolet light (UV) for 30–60 min before cell seeding.

4.4.1 Culture conditions of mouse embryonal fibroblasts in Study I

Mouse embryonal fibroblasts (MEFs) originally obtained from Wolfgang H. Ziegler (Hannover Medical School, Hannover, Germany) were cultivated in DMEM high glucose, w/o L-glutamine, w/o sodium pyruvate (Biowest, L0101) medium supplemented with 10 percent (v/v) fetal bovine serum (FBS; American fetal bovine serum, Biowest S1520), 1 percent L-glutamine (Biowest, X0550), 1 percent antibiotic penicillin-streptomycin (Biowest L0022; 100 IU/mL penicillin, 100 µg/mL streptomycin) in a humified atmosphere of 95 percent air and 5 percent CO$_2$ at 37°C. Cell experiments were performed in passages 15-20. Cells were seeded on studied CNF surfaces and a glass cover slip was used as a control material.
4.4.2 Culture conditions of adipose derived stem cells and human dermal fibroblasts in Study II

NHDFs (Lonza, Basel, Switzerland, Cat. no. C-2509) were cultivated in DMEM supplemented with 10 percent FBS (Thermo Fisher Scientific, Gibco, Waltham, MA, Cat. no. 10270-106) and 40 µg/mL gentamicin (LEK, Ljubljana, Slovenia).

ADSCs were isolated from lipoaspirates after confirmed written informed consent from donors, in compliance with the Declaration of Helsinki, and under ethical approval by the Ethics Committee at Na Bulovce Hospital in Prague. The isolation procedure according to Estes et al. (Estes et al. 2010) were performed, with slight modifications as previously described. (Bacakova et al. 2018; Travnickova et al. 2020) The pooled ADSCs were cultivated in DMEM supplemented with 10 percent FBS, 40 µg/mL gentamicin, and 10 ng/mL recombinant human basic fibroblast growth factor (FGF2; GenScript, Piscataway, NJ).

The CNF-coated and noncoated meshes were fixed into CellCrown inserts and inserted in 24-well plates, and were seeded with NHDFs and ADSCs in Passage 3 at a density of 20000 cells/insert (that is, approximately 25000 cells/cm²) in 1 mL of the cell culture medium. An amount of 0.5 mL/well was added after 2 h of cultivation into the final volume of 1.5 mL/well. The cells were cultivated for seven days in a cell incubator at 37°C in a humified air atmosphere with 5 percent CO₂. Polystyrene (PS) of the culture plates was used as a control material.

4.5 Cell characterization methods

AlamarBlue Cell Viability Assay Reagent (G-Biosciences) was used to study the level of cellular metabolic activity and to evaluate the number of viable MEF cells in Study I. In Study II, the level of metabolic activity of the NHDFs and ADSCs on studied materials was measured on Days 1, 3, and 7 using the conversion of resazurin sodium salt (Sigma-Aldrich) into resorufin. Details of the protocols can be found in the original publication (Study I and Study II, respectively).

In Study I, cell proliferation was studied using cell counts determined from OM images, and cell morphology from OM images obtained during real-time imaging. In Study II, the morphology of the cells was studied using fluorescence microscopy (by staining filamentous actin and vinculin) and SEM imaging. Vinculin staining was used to indicate the level of specific receptor-mediated cell adhesion and cell spreading.
4.6 Quantification of cell and cellulose nanofiber alignment

Cell alignment was studied in Study I and CNF alignment in Studies I and III. A spectral orientation analysis tool CytoSpectre (Kartasalo et al. 2015) was used to characterize the degree of alignment (Studies I and III), and the mean orientation angle (Study III). CytoSpectre is a software tool used for the analysis of orientation and wavelength distributions from micrographs. The software utilizes the Fourier transform to estimate the power spectrum of an image and based on the spectrum, computes parameter values describing, among other things, the mean orientation and isotropy.

In Study I, circular variance (CV) values were used to determine the degree of cell alignment and the degree of fiber alignment. Cell alignment was measured from images obtained with live cell imaging and fiber alignment from AFM amplitude images of the CNF surfaces. CV is a measure of the isotropy of the orientation distribution. A value of zero corresponds to perfect anisotropy – that is, orientation, while a value of one corresponds to perfect isotropy – that is, lack of orientation. Orientation plots were obtained to point out the orientation direction in the example images.

In Study III, the CNF alignment was evaluated from SEM images taken from the surface of anisotropic self-assembled and isotropic control samples. SEM images at different locations on the film surfaces were used for the orientation analysis. The CV value was determined from each SEM image. In addition, a mean orientation angle of each location was determined for the evaluation of orientation direction along the evaporating boundary line.

4.7 Statistical analyses

In Study I, the data analysis was performed with Matlab Software by calculating average and standard deviation (SD) values for the data obtained from AFM images. The calculated parameters included an average high spot count (HSC) ($n = 256$) and an average wavelength ($\lambda_a$). Both parameters were calculated for all vertical ($n = 256$) and horizontal ($n = 256$) roughness plots. In addition, average values and standard deviations were calculated for proliferation data, cell viability data, and circular variances.

In Study II, the statistical significance of the AFM data was evaluated using the nonparametric analysis of variance (Kruskal–Wallis), with Tukey’s posthoc test for
pairwise comparison. Values of $p \leq 0.05$ were considered significant. The data postprocessing and statistical testing were performed in Matlab, if not stated otherwise. The parametric data, including those of swelling ratio measurements, the cell metabolic activity, and the protein adsorption, were evaluated using parametric analysis of variance (ANOVA) with Tukey’s posthoc test for pairwise comparison. Values of $p \leq 0.05$ were considered significant. Cell metabolic assay data were presented as the arithmetic mean ± standard deviation and were used to construct growth plots to view the overall growth dynamics of the cells.

In Study III, Matlab software was used to calculate average values for resistance measurement data of the parallel films and the repeat measurements ($n=9$) with a 95 percent confidence interval.
This chapter summarizes the main results of Studies I–III. Study I investigated CNF alignment (Section 5.1.1 and 5.1.2), developed surfaces with aligned CNFs (Section 5.1.2), and investigated cell growth and cell orientation (Section 5.4.1) on the aligned surfaces. In Study II, CNF-coated meshes were developed, their properties were investigated (Section 5.2.2), and cell behavior on anionic and cationic CNF-coated cellulose meshes was investigated (Section 5.4.2). In Study III, a method was developed to align c-CN in the presence of MWCNT component to obtain electrically anisotropic nanocomposite films (Section 5.1.3). The properties of the control isotropic and these anisotropic nanocomposite films (Section 5.2.3) and interactions of nanomaterial components were examined (Section 5.3.2).

5.1 Evaporation-induced self-assembly of cationic cellulose nanofiber

Evaporation-induced self-assembly was used in Studies I and III in order to develop surfaces with aligned CNF (Study I) and electrically anisotropic nanocomposite films (Study III). Droplet evaporation mechanism (Section 5.1.1) and alignment of c-CN along evaporating boundary line (Section 5.1.2) were investigated in Studies I and III. The theory of evaporation-induced self-assembly is discussed in Chapter 6.

5.1.1 Droplet evaporation mechanism

There are two possible mechanisms for the evaporating boundary line (Study I): the boundary line either pins or depins during evaporation. A pure pinning of the boundary line causes the formation of a “coffee ring” deposit, while the altering of pinning and depinning results in the formation of concentric rings. To study whether the evaporation boundary line is pinned or unpinned during evaporation, droplet evaporation of a c-CNF suspension was imaged under an optical microscope.
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5.1 Evaporation-induced self-assembly of cationic cellulose nanofibers

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Figure 4. Side profiles (a–c) and top view images (d–f) of evaporating droplets. Snapshots captured during evaporation using goniometer (side profiles) or inverted microscope (top view): (a and d) c-CNFS on PVAm, (b and e) deionized water on PVAm, and (c and f) deionized water on glass. Adapted with permission from A. Skogberg et al. (2017), Biomacromolecules, 18(12), p. 3936-3953, DOI: 10.1021/acs.biomac.7b00963. Copyright 2017 American Chemical Society.
5.1.2 Alignment of cationic cellulose nanofibers along the boundary line

Study I investigated the degree of orientation of CNFs on evaporated surfaces. AFM images (Figures 6 and 7) were used to obtain information on the fiber arrangement and surface topography of the droplet-evaporated surfaces. Figure 6 presents the results of spectral orientation analysis, in which the degree of anisotropy was analyzed from AFM images. In spectral analysis, evaporated u-CNF (Figure 6a), a-CNF (Figure 6c), and c-CNF (Figure 6e) surfaces were imaged and analyzed. Orientation plots (Figure 6b, d, and f) present the mean orientation angle and confirm the orientation direction, while CV values (Figure 6g) describe the degree of orientation. c-CNF and u-CNF surfaces showed higher anisotropy compared to more isotropic a-CNF surfaces.
Figure 5. Time evolutions of contact angle and the diameter of the droplet-surface interface (mm) analyzed using goniometer recordings in the case of (a) c-CNFS droplet (n = 6) evaporation on PVAm-coated glass, (b) \(dH_2O\) droplet (n = 6) evaporation on PVAm-coated glass, and (c) \(dH_2O\) droplet (n = 6) evaporation on glass substrate. Distances of droplet edges from the center point (d–f) analyzed using OM recording in the case of (d) c-CNFS droplet evaporation on PVAm-coated glass, (e) \(dH_2O\) droplet evaporation on PVAm-coated glass, and (f) \(dH_2O\) droplet evaporation on glass substrate. Distances are analyzed from points located at the right and left sides of the droplet edges. Adapted with permission from A. Skogberg et al. (2017), Biomacromolecules, 18(12), p. 3936-3953, DOI: 10.1021/acs.biomac.7b00963. Copyright 2017 American Chemical Society.

Figure 6. AFM amplitude images of (a) u-CNFS (1 μm × 1 μm), (c) a-CNFS (1.5 x1.5 μm), and (e) c-CNFS (1.5 × 1.5 μm) surfaces show the arrangement of CNFs on the droplet-evaporated surfaces. Orientation plots of (b) u-CNFS, (d) a-CNFS, and (f) c-CNFS surfaces are obtained from the AFM images using the analyzing software (Cytospectre). (g) Average CVs (n = 3) describe the isotropy (degree of orientation). Value zero corresponds to perfect alignment of all oriented structures along single line. Value one corresponds to a perfect lack of a dominant orientation. Reprinted with permission from A. Skogberg et al. (2017), Biomacromolecules, 18(12), p. 3936-3953, DOI: 10.1021/acs.biomac.7b00963. Copyright 2017 American Chemical Society.

Relevant to cell studies in Study I, alignment of c-CNFS along the evaporating boundary line was investigated in more detail using AFM (Figure 7a–b, d–e), OM
In the spectral analysis, higher anisotropy was found in locations closer to the evaporated c-CNF surface edge (Figure 7a-b), while c-CNF surfaces close to the center area were more isotropic (Figure 7d-e). The degree of orientation on the peripheral area (Figure 7c) of the evaporated c-CNF surface was detected with an average circular variance of 0.27. On the center area (Figure 7c) the average circular variance was 0.64. Alignment was also visible in OM (Figure 7f) and HIM (Figure 7g) images. The topic is covered in more detail in the discussion (Chapter 6).

Figure 7. AFM amplitude images (1.5 x 1.5 µm in (a) and (d), 1 x 1 µm in (b) and (e)), OM image (f) and HIM (g) image from the peripheral area (a, b, f, g) and center area (d, e) of the sketched (c) evaporated c-CNF droplet. Images are from separate evaporated droplets with volumes of 2–200 µL. Fibers are oriented in the peripheral areas, while the center area has a lower degree of orientation. Adapted with permission from A. Skogberg et al. (2017), Biomacromolecules, 18(12), p. 3936-3953, DOI: 10.1021/acs.biomac.7b00963. Copyright 2017 American Chemical Society.
In addition to the spectral analysis of the orientation, c-CNδ alignment was evaluated using a quantitative analysis (Figure 6 of Study I) of surface roughness plots obtained from AFM images of anisotropic c-CNδ (Figure 6a of Study I) and isotropic a-CNδ (Figure 6c of Study I) surfaces. Isotropic a-CNδ – with a lower degree of orientation – was used as a control surface. Surface roughness plots were obtained from AFM images of the aligned c-CNδ surface (Figure 6b of Study I). Plots were different between the directions parallel to the assumed fiber orientation (horizontal) and perpendicular to the fiber orientation (vertical). Despite that, surface roughness (measured as Ra) was similar in both directions. However, the peaks in the roughness plot appeared more frequently perpendicular to the assumed alignment than parallel to the assumed alignment (Figure 6b of Study I).

5.1.3 Cationic cellulose nanofiber -driven assembly of nanomaterial components

In Study III, evaporation-induced self-assembly of c-CNδ along the evaporating boundary line was utilized in fabrication of electrically anisotropic nanocomposite films using c-CNδ and MWCNT. Based on findings in previous studies, Study I, and (Siljander et al. 2018), we presented a hypothesis for explaining the structural composition and the electrical conductivity of the films fabricated from a dispersion of c-CNδ and MWCNT (control) or suspension of this dispersion with suspended additional c-CNδ. Figure 8 presents this hypothesis, which assumes that an isotropic conductive film results when water from the hes-c-CNδ/MWCNT dispersion is evaporated, and anisotropic film results when water is evaporated from a suspension composed of the hes-c-CNδ/MWCNT dispersion and additional c-CNδs.
Figure 8. Skogberg, A., et al. (2022) Conceptual images of the hypothesized structure of nanocomposite films (b, c, e, f, and g). Left side (b and e) represents the control film manufactured using the hes-c-CNF/MWCNT dispersion. Right side of (c and g) represents the assembled nanocomposite film manufactured using the c-CNF + hes-c-CNF/MWCNT suspension. The schematic represents simulations of electrical properties of an isotropic control (a) and anisotropic self-assembled (d) nanocomposite films using finite element method (FEM). Alignment direction of free assembling c-CNFs is shown in (g). Light grey represents c-CNFs, while dark grey is MWCNTs. In the control film (e) dark grey MWCNTs are surrounded by the c-CNF matrix (white surroundings). During dispersion preparation, i.e. sonication, c-CNFs are fibrillated and degraded in length such that they cover the MWCNT surface (f). The added c-CNFs during suspension preparation are larger in size compared to hes-c-CNF. The repeating, alternating structure in (g) is an oversimplification. The illustrations are oversimplified and not in scale and do not try to show the actual sizes or actual structure of the assembled film but give an overview of the hypothesized repeating structure at the nanoscale, and show the difference between control and assembled films. [Figure]. In Self-assembled cellulose nanofiber–carbon nanotube nanocomposite films with anisotropic conductivity. Nanoscale, 14. Retrieved from doi.org/10.1039/D1NR06937C.

As discussed in Section 5.1.2, CNFs align along the evaporating boundary line. In Study III, the c-CNF alignment was investigated in the presence of MWCNT. SEM images were analyzed using CytoSpectre software in order to show the assumed higher anisotropy in the assembled samples S1–S3 compared to the isotropic control samples. According to the SEM study, the MWCNT does not align in any of the studied nanocomposite films.

Orientation analysis was also performed using the circular variance values obtained with CytoSpectre. c-CNF+hes-c-CNf/MWCNT films were more
anisotropic compared to more isotropic hes-c-CNF/MWCNT. Figure 9 presents the circular variance values, which are remarkably different between the control and the assembled samples S1–S3. The circular variance averages (n=4) of the films S1, S2, S3, and Control are 0.76, 0.65, 0.67, and 0.96, respectively. All the circular variance values of the assembled nanocomposite films are higher than the values determined for the c-CNF films (0.27) in Figure 6e–g. This is an expected result as, in the assembled nanocomposite films, the c-CNF is the aligning component, while MWCNT was more randomly oriented.

Figure 10 explains the orientation analysis presented in Figure 9 in more detail. Individual c-CNFs (Figure 10b) from c-CNF + hes-c-CNF/MWCNT suspension align according to hypothesis, while hes-c-CNF/MWCNT is more random. Individual aligned c-CNF pack and aggregate close together during alignment and evaporation, and it is not possible to distinguish separate fibers from SEM images, such as in Figure 10a. However, the scans from the surface show the expected alignment direction when analyzed using spectral analysis (Figure 10a and d). The example image in Figure 10a was scanned in the specific location presented in Figure 10c and is expected to have c-CNF alignment along the evaporating triple line. The triple line moves very symmetrically from the edge towards the center during evaporation. Spectral analysis confirm that the orientation angle (Figure 10d) is to the expected orientation direction, that is, along the evaporating boundary line.
Figure 9. Skogberg, A., et al. (2022) Low vacuum SEM scans from the surface of the S3 (a) and control (b) films. Scale bar in a and b is 50 µm. Examples of scanning locations (c) and the corresponding mean orientation angles (e) of the assembled films. The mean orientation angles for the control films are not presented, as the circular variance is close to 1 in all scanning locations of the control films (d), so no primary alignment direction is expected. A graphical illustration (f) of the orientation describes the orientation direction in Scanning Point 2 (SEM image in (a)), while isotropy (g) is confirmed for the location seen in SEM image (b). Circular variances of the films S1, S2, S3, and control (h). Circular variance averages (n = 4) or the films S1, S2, S3, and control are 0.76, 0.65, 0.67 and 0.96, respectively. Perfect isotropy corresponds to circular variance value 1, while 0 stands for perfect anisotropy. [Figure]. In Self-assembled cellulose nanofiber–carbon nanotube nanocomposite films with anisotropic conductivity. Nanoscale, 14. Retrieved from doi.org/10.1039/D1NR06937C.
5.2 Substrate and coating properties

Modified CNF grades were used to prepare different coatings and cell cultivation substrates in Studies I and II. This section summarizes the main results between different types of coatings and different CNF grades.

5.2.1 Roughness properties of the substrates and coatings in Study I

In Study I, CNFs were used for the preparation of surfaces for cell growth and orientation. The arithmetic average height parameter (\(R_a\)) value describes the overall roughness of the studied surface. In Study I, the \(R_a\) value was determined in the peripheral and center areas of the droplet-evaporated surfaces. The \(R_a\) values of 4.52–5.68 of all the three CNF surfaces in the peripheral location of the evaporated droplets indicate relatively smooth surfaces. Slightly higher \(R_a\) values of
approximately 11 were obtained in the center area possessing more isotropic surface topography. The dynamic contact angles of the u-CN, a-CN and c-CN surfaces used for the cell experiments were 32.56, 29.64, and 67.70, respectively.

### 5.2.2 Properties of the substrates and coatings in Study II

In Study II, charged CNF were used as biocompatible coatings of cellulose meshes for improved cell adhesion. \(R_s\) median measured on multiple regions of a600, c600 and c+a-CNF-coated meshes was 9.04 nm, 10.20 nm, and 55.76, respectively. Groups c600 and a600 were significantly different from group c+a (\(p = 0.025\)).

Contact angles and swelling of the coatings were determined using water and DMEM. Contact angles of the c600, a600, and c+a-CNF-coated glass coverslips measured with dH\(_2\)O were 65°, 31°, and 32°, respectively, while the corresponding values measured with DMEM were 50°, 40°, and 32°, respectively. The swelling ratio of the coatings in DMEM was higher in c600 coatings than the a600 and c+a coatings after 20 min in DMEM. After 20 h, there was no additional water uptake by the c600 coatings, while that of the a600 coatings increased with time. Increase in swelling ratio between 20 min and 20 h time points in the case of c+a coatings was lower than in the a600 coatings. There were no significant differences in the swelling ratios of the noncoated meshes between time points or between DMEM and dH\(_2\)O.

According to the micro-CT analysis, the non-coated meshes had a high porosity of 84.9 percent with an average fiber thickness 7.2±1.97 \(\mu\)m and an average void thickness 44.3±36.3 \(\mu\)m. The coated meshes were categorized either as 2D or 3D coatings according to the appearance of the SEM surface and the cross-section scans. Depending on the charge and volume of the applied CNF, the coating suspensions either penetrated into the pores of the mesh (3D) or formed a layer on top of the mesh (2D), described in more detail in Study II. The best 3D microtopography coating was fabricated using 150 \(\mu\)L of a-CNFs, while the most film-like 2D coating was obtained with 600 \(\mu\)L of c-CNFs or c+a-CNFs.

The average stiffness values (arithmetic mean ± SD) of the dry c600, a600, and c+a-CNF-coated meshes were 0.572 ± 0.24 GPa (Mdn = 0.640), 0.683 ± 0.45 GPa (Mdn = 0.538), and 0.315 ± 0.10 GPa (Mdn = 0.275), respectively. After wetting the coated meshes in DMEM for 20 min, the stiffness of the corresponding samples dropped and was, on average, three orders of magnitude lower than that on dry samples: 121 ± 16 kPa (Mdn = 116), 342 ± 101 kPa (Mdn = 326), and 241 ± 83 kPa (Mdn = 230), respectively. This indicates a considerable softening of the substrates.
after wetting. The stiffness of the c600 was the lowest, followed by that of the c+a coatings; the highest stiffness was measured from the a600. After 17 h in DMEM, the stiffness of only a600 decreased significantly compared to that measured after 20 min, while those of c600 and c+a coatings did not change significantly. This was in accordance with the swelling ratios. In the case of a600 coatings, the stiffness decreased and the water uptake increased with time, which means that a600 coatings become softer during incubation in DMEM.

5.2.3 Electrical properties of the nanocomposite films (Study III)

The c-CNF+hes-c-CNF/MWCNT films showed anisotropic electrical properties, while the hes-c-CNF/MWCNT films showed more isotropic electrical properties. Figure 11 includes a schematic representation, in which left side of the figure presents the isotropic film and the right side presents the anisotropic film. Figure 11 includes IR images, which is consistent with the results of electrical measurements presented in Figure 12. SEM images in Figure 11 represent the structural differences of the isotropic and anisotropic films.

A difference was observed on the film surfaces of the evaporated hes-c-CNF/MWCNT (Figure 11, left-hand side) and c-CNF+hes-c-CNF/MWCNT (Figure 11 right-hand side) films. IR images in Figure 11 (top) depict the anisotropic electrical properties of the c-CNF+hes-c-CNF/MWCNT (top right) films at the macroscopic scale, and more isotropic properties of the hes-c-CNF/MWCNT (top left) films. In the isotropic film the heat flows through the fastest route; that is, through the center of the film and spreads evenly (not shown). In the anisotropic film, the center area shows a lower temperature, indicating that the current is restricted to pass through the center line, which is the shortest path from terminal to terminal. The electrical anisotropic properties are presented in more detail in Figure 12.

The SEM characterization was focused on the edges of the isotropic control hes-c-CNF/MWCNT film and the anisotropic c-CNF+hes-c-CNF/MWCNT film (Figure 11, bottom right) because we previously reported that the self-assembly of c-CNFs begins on the droplet boundary line. SEM characterization of the film edges shows that, in the isotropic control film (Figure 11 bottom left), the edges are not as uniform as in the anisotropic films.
Resistance measurements confirm the finding obtained with IR-imaging: remarkable differences are shown in conductivity between the isotropic control and the anisotropic assembled films S1-S3. The resistance measurement results are presented in Figure 12. The isotropic control hes-c-CNFMWCNT films show relatively constant resistance throughout the film, while the anisotropic c-CNFMWCNT films (S1–S3) show evident anisotropy (Figure 12). There is also significant difference in resistance along the center line compared to the resistance along the circular zones of the anisotropic films, while less difference is shown between the corresponding directions in isotropic control films. The resistance increases along the center line when moving towards the center (Measurement A and C), and also when measured along the circles (Measurement B). The increase in resistance in Measurement B is remarkable, although the probe distance decreases, when moving towards the center along the zones. In contrast in isotropic control films, the resistance decreases slightly along the circles (Measurement B) when moving towards the center and the distance decreases. Resistance measured along
Circles 1 and 3 (Measurement D and E) shows relatively even conductivity in all measured films.

To study the repeatability of the measurements and fabrication of the films, the measurements were repeated at least three times and three parallel films of each sample type were measured (Figure 13). All the measurements were repeated at least three times to investigate the stability of the films. Each nanocomposite film was applied in the electric field for at least 11 min during the resistance measurements. In addition, three parallel films were fabricated in order to show the repeatability of the film manufacture. In addition, different voltages between the measurements were used. Similar heating with equal voltages were observed once the heating was repeated. This shows that application of an electric field does not change the properties of the film.

To study the long-term stability of the nanocomposite films, the same films were measured and imaged repeatedly during a time span of approximately half year. A nanocomposite film was heated and IR imaged on different days in the long-term stability experiment, during which the sample showed consistent heating without changes in its properties (more detailed results are provided in Study III). As a summary, the anisotropic nanocomposite structure was considered stable both during time and when the electric field is applied.
Figure 12. Skogberg, A., et al. (2022) Resistance of the assembled (S1–S3) and control (C) films. Resistance measurement protocol (presented at the upper row). Results for films S1–S3 and control from locations along the radial line (measurements A–C presented at the middle row) and along circles 1 and 3 (measurements D and E, at the lower row). In measurements, locations n_n–p_n, n_n and p_n stand for negative and positive probe locations, respectively. [Figure]. In Self-assembled cellulose nanofiber–carbon nanotube nanocomposite films with anisotropic conductivity. Nanoscale, 14. Retrieved from doi.org/10.1039/D1NR06937C.
5.3 Interactions and chemical characterization of nanomaterial components

To obtain more information regarding the effects of sonication treatments and interaction between c-CNF and MWCNT, MAS-NMR spectroscopy was applied in Study III.

The $^1$H NMR spectra illustrate the hydrogen peaks of the untreated c-CNF (Figure 14a). The untreated c-CNF was used as a reference for the investigation of the effects of sonication treatment on hes-c-CNF (Figure 14b) and on hes-c-CNF-MWCNT dispersion (Figure 14c). The intensity and the area of the signal peak at 2.1 ppm results from –CH$_3$ groups of the functionalized hydroxypropyltrimethylammonium side chain and remain relatively unchanged in c-CNF samples (Figure 14a–b). This indicates that the functional group remains intact and relatively stable throughout the sonication treatments. Untreated c-CNFs and c-CNFs sonicated with 625 kJ g$^{-1}$ provide the same signals. Therefore, it is assumed that there is no significant effect of sonication on the chemical structure of c-CNF. It is not straightforward to evaluate the interaction between c-CNFs and MWCNTs during sonication from $^1$H NMR spectra (Figure 14c). This is because the analyte is merged and signals are broad and overlapping. However, compared to the signals obtained from c-CNF alone, the following conclusion can be drawn on the organization of c-CNFs in the sample: the functional group is expected to point out from the entangled hes-c-CNF-MWCNT nanostructure. As the peaks in 5 ppm to 3.8 ppm are weak and broadened, the corresponding protons are not solvated. This
indicates strong interaction between those areas of c-CNFs with MWCNTs. The nanostructure formed during sonication is tightly packed, preventing water from penetrating between the interaction sites. In summary, as the trimethyl protons of the cationic substituent remain unchanged in studied samples, the trimethyl ammonium group is assumed to face outward from MWCNTs. On the other hand, the less solvated area refers to cellulose ring protons; in this case, one of H3–H5. This is suggested to be the location of the stronger interaction with MWCNTs.

Therefore, according to MAS-NMR, the interaction between c-CNFs and MWCNT molecules is strong. In addition, we obtain valuable information about the trimethyl ammonium group of c-CNFs, which points outwards of the c-CNFS/MWCNT cluster and is therefore able to interact with surrounding molecules. The meaning of this finding is discussed in more detail in Chapter 6.
indicating strong interaction between those areas of c-CNFS with MWCNTs. The nanostructure formed during sonication is tightly packed, preventing water from penetrating between the interaction sites.

In summary, as the trimethyl protons of the cationic substituent remain unchanged in studied samples, the trimethyl ammonium group is assumed to face outward from MWCNTs. On the other hand, the less solvated area refers to cellulose ring protons; in this case, one of H3–H5. This is suggested to be the location of the stronger interaction with MWCNTs. Therefore, according to MAS-NMR, the interaction between c-CNFS and MWCNT molecules is strong. In addition, we obtain valuable information about the trimethyl ammonium group of c-CNFS, which points outwards of the c-CNFS/MWCNT cluster and is therefore able to interact with surrounding molecules.

The meaning of this finding is discussed in more detail in Chapter 6.

**Figure 14.** The FG-MAS 1H NMR spectra of the untreated c-CNFS (a), hes-c-CNFS supernatant sonicated 625 kJ/g (b), and hes-c-CNFS-MWCNT dispersion sonicated 625 kJ/g (c). Adapted from "Self-assembled cellulose nanofiber–carbon nanotube nanocomposite films with anisotropic conductivity" by A. Skogberg et al. (2022), Nanoscale, 14, p. 10 in Supplementary Information. Open Access license cc BY-NC.
5.4 Cell responses to charged cellulose nanofibers

Cell responses to a-CNf and c-CNf were explored in Study I and II. The objective in Study I was to obtain cell orientation on the aligned c-CNf surfaces and study the viability of the cells on different CNF surfaces (Section 5.4.1), while the goal in Study II was to achieve enhanced cell viability (Section 5.4.2) on CNF-coated commercial cellulose meshes, which are potential cell carriers.

5.4.1 Cell proliferation, viability and cell orientation (Study I)

Cell growth and viability were investigated on evaporated u-CNf, a-CNf, and c-CNf surfaces and the corresponding surfaces coated with fibronectin (FN). Compared to u-CNf and negative control with 10 percent DMSO, the cell growth was significantly improved on the positive control glass cover slip, a-CNf, and c-CNf surfaces, as well as on the corresponding FN-coated surfaces (details in Figure 14 in Study I). MEFs on the oriented c-CNf surface showed the highest proportional increase in cell number, comparable to the values obtained for the positive FN-coated control, followed by FN-coated a-CNf, FN-coated c-CNf, positive control, a-CNf, and c-CNf surfaces. The cell numbers were slightly higher in all FN-coated surfaces than the corresponding surfaces without the FN coating, indicating slight enhancement in cell proliferation on FN-coated samples. Viability of MEFs on different CNF surfaces was investigated using the AlamarBlue assay. The AlamarBlue results showed similar trend to the one found with the proliferation data. The highest viability was found on a-CNf surfaces. In all tested sample types, the cell viability, as well as proliferation, was higher in the FN-coated samples compared to the uncoated samples.

MEFs cultured on the positive glass control surface presented notably adhered morphology without orientation (Figure 15 a and c). Cells cultured on a-CNf surfaces had similar non-oriented adhered morphology (not shown here), while the cell morphology on the c-CNf surfaces showed remarkable elongation (Figure 15 b and d). The cell morphology on FN-coated surfaces were similar to those on the corresponding uncoated surfaces.
Cell responses to charged cellulose nanofibers were explored in Study I and II. The objective in Study I was to obtain cell orientation on the aligned c-CNFS surfaces and study the viability of the cells on different CNF surfaces (Section 5.4.1), while the goal in Study II was to achieve enhanced cell viability (Section 5.4.2) on CNF-coated commercial cellulose meshes, which are potential cell carriers.

5.4.1 Cell proliferation, viability and cell orientation (Study I)

Cell growth and viability were investigated on evaporated u-CNFS, a-CNFS, and c-CNFS surfaces and the corresponding surfaces coated with fibronectin (FN). Compared to u-CNFS and the negative control with 10 percent DMSO, the cell growth was significantly improved on the positive control glass cover slip, a-CNFS, and c-CNFS surfaces, as well as on the corresponding FN-coated surfaces (details in Figure 14 in Study I). MEFs on the oriented c-CNFS surface showed the highest proportional increase in cell number, comparable to the values obtained for the positive FN-coated control, followed by FN-coated a-CNFS, FN-coated c-CNFS, positive control, a-CNFS, and c-CNFS surfaces. The cell numbers were slightly higher in all FN-coated surfaces than the corresponding surfaces without the FN coating, indicating slight enhancement in cell proliferation on FN-coated samples.

Viability of MEFs on different CNF surfaces was investigated using the Alamar Blue assay. The AlamarBlue results showed similar trend to the one found with the proliferation data. The highest viability was found on a-CNFS surfaces. In all tested sample types, the cell viability, as well as proliferation, was higher in the FN-coated samples compared to the uncoated samples. MEFs cultured on the positive glass control surface presented notably adhered morphology without orientation (Figure 15a and c). Cells cultured on a-CNFS surfaces had similar non-oriented adhered morphology (not shown here), while the cell morphology on the c-CNFS surfaces showed remarkable elongation (Figure 15b and d). The cell morphology on FN-coated surfaces were similar to those on the corresponding uncoated surfaces.

The orientation of the cells on studied CNF surfaces were examined using an optical microscope and spectral orientation analysis (Figure 16). Cells aligned in a circular pattern on the droplet-evaporated c-CNFS surfaces (Figures 17a-b). Cell alignment is visible in the peripheral areas of the circular c-CNFS surfaces, while cells in the central area remain in a random orientation (Figure 17b-c). This accords with suggested c-CNFS alignment parallel to the evaporation boundary line, while more isotropic c-CNFSs were observed in the center of the dried c-CNFS surface.

To control the orientation direction of c-CNFS, contact-dispensing was used to make a straight c-CNFS line, which was let to evaporate. This resulted in aligned c-CNFSs and correspondingly unidirectionally aligned cells parallel to the c-CNFS evaporation line axis (Figure 16c). The proliferation and elongation of the cells parallel to the boundary line are shown in Videos F and G (associated content of Study I), which was reconstructed from the microscope images during the 48h cultivation period. The possibility of controlling the orientation direction of the cells is one of the key findings of Study I.
The cell orientation was analyzed using spectral analysis (Figure 16). No significant degree of orientation was detected on the a-CNF surface or on the positive control surface. The average circular variance values were 0.89 (n = 19) on a-CNF and 0.93 (n = 9) on positive control (Figure 16a). On the c-CNF surfaces, by contrast, the orientation is clearly visible in the microscope images, confirmed by spectral analysis, with average circular variance value of 0.39 (n = 33; Figure 16a). In addition to the CV values, Figure 16 presents example images with randomly oriented cells on the control surface (Figure 16b) and oriented cells on the c-CNF surfaces (Figure 16c), and the corresponding schematic orientation plots provided by the Cytospectre software (Figures 16d and e, respectively). Figure 17b shows the droplet-evaporated c-CNF surface and the oriented cells along the evaporated droplet shape, including an example of more oriented peripheral cells (Figure 17a) and more randomly distributed cells in the central part of the dried area (Figure 17c).

**Figure 16.** Cell morphology on positive control (glass) and elongated cell morphology on aligned nanocellulose one day after plating the cells. Adapted with permission from A. Skogberg et al. (2017), Biomacromolecules, 18(12), p. 3936-3953, DOI: 10.1021/acs.biomac.7b00963. Copyright 2017 American Chemical Society.
The viability of NHDFs and ADSCs on the CNF-coated and noncoated meshes was investigated in Study II at three time intervals by measuring the cell metabolic activity using the resazurin assay. The cell growth on a-CNF- or c+a-CNF–coated meshes were comparable to that on standard tissue culture PS. However, the viability was similar between c-CNF-coated meshes and noncoated meshes. These differences in viability were more apparent in NHDFs than in ADSCs.

In addition to viability measurement, the cell morphology was visualized from fluorescent images on each time point (Day 1, Day 3, and Day 7). Each time point also had more detailed focus, as described next. The initial adhesion of both studied cell types on the CNF-coated and noncoated meshes on Day 1 (Figure 3 in Study II) and the adsorption of serum-derived proteins (Figure 4 in Study II) were examined. The proliferation and the morphology (Figures S5 in Study II) of the cells were observed on Day 3, and on Day 7 the final status of the colonization of the CNF-coated cellulose meshes with cells was evaluated (Figures S6 and 6 in Study II).

On Day 1, the initial adhesion of cells on CNF-coated meshes was different between NHDFs and ADSCs (Figure 3 in Study II). The metabolic activity of the NHDFs was significantly lower on the c-CNF coatings than that on the a-CNF and c+a-CNF coatings, while the metabolic activity of ADSCs on the c-CNF coatings was mostly similar to, or even slightly better than it was on the a-CNF coatings. The cell metabolic activity of both cell types on c+a-CNF coated meshes was almost on
the same level as on polystyrene control. The cell morphology of NHDFs on c600-CNFCNF-coated samples was round and nonspread, indicating poor adhesion. The adhesion and spreading of ADSCs were almost the same as those on the a600-CNFCNF- and c+a-CNF-coated meshes, indicating good adhesion of ADSCs on all studied surfaces. The 3D topography of the a150-CNF coated meshes supported the adhesion and elongation of both cell types, while the more flat c600-, a600-, and c+a-CNF coatings provided the cells with a more homogeneous flat area. This resulted in cell spreading into a polygonal shape indicating good adhesion.

On Day 3, cell growth and morphology were investigated. Figure 18 presents the SEM images used to visualize cell morphology. The a-CNF-coated meshes (a150, a600) remarkably increased the proliferation of both cell types compared to the noncoated meshes and the c-CNF-coated meshes (c150, c600). The negative influence of c-CNFSs on cell behavior became visible also in ADSCs, although their metabolic activity was still slightly higher on the c150- and c600-CNF-coated meshes than the metabolic activity of the NHDFs. The metabolic activity of both cell types on the c+a-CNF-coated meshes was comparable with the values on the control polystyrene. However, it was remarkably lower than on the a600-CNF coated meshes. The flat 2D surface of the c600-CNF coated mesh supported the growth of ADSCs, while the NHDFs began to detach from the c600 coatings (Figure 18). On the 2D surfaces, the number of both cell types was higher on the a600-CNF coatings. Compared to 2D surfaces, more elongated cells were observed on the 3D surface of the a150 coatings. Both cell types were well spread on the combination coating c+a-CNFCNF and dividing cells were visible, such as ADSCs on c+a in Figure 18. The cells on noncoated meshes were round and poorly attached (Figure 18).

On Day 7, the metabolic activity of ADSCs on all studied surfaces was lower than the metabolic activity of the NHDFs. However, the proliferation of both cell types was significantly higher on the a-CNF- and c+a-CNF-coated meshes than those on the c-CNF-coated and noncoated meshes. The numbers of both cell types were higher on c150 than those on c600. On a600 and c+a coated meshes; both cell types were confluent. On Day 7, the cell viability on the 3D and 2D surface of a150- and a600-CNF-coated meshes, respectively, was comparable, especially in the case of NHDFs. The 2D coatings (a600 and c+a) enhanced the proliferation and spreading of both cell types only in the xy directions, while the 3D coating (a150) supported elongation of the cells on the mesh fibers and between them in all xyz directions (Figure 6 in Study II) and thus provided more space for cell elongation and proliferation. The negative effect of c600 coatings was shown as the formation of clusters of ADSCs and spheroids of NHDFs. A positive effect of the 3D topography
of the a150 coatings and also of the c150 coating on cell growth was observed during one week of cultivation.

![A Morphology of NHDFs](image1.png)

![B Morphology of ADSCs](image2.png)

**Figure 18.** Pajorova, J., et al. (2020) Morphology of NHDFs (A) and ADSCs (B) on CNF-coated and noncoated meshes on day 3 after cell seeding, acquired by SEM. Scale bar = 20 μm. [Figure]. In Cellulose Mesh with Charged Nanocellulose Coatings as a Promising Carrier of Skin and Stem Cells for Regenerative Applications. Biomacromolecules, 21(12). Retrieved from doi.org/10.1021/acs.biomac.0c01097.
6 DISCUSSION

6.1 Alignment of cationic cellulose nanofibers during evaporation

This thesis work has shown that c-CNF align along the evaporating boundary line. CNF, due to its nanoscale dimensions is extremely difficult to visualize, especially in suspension state, when individual CNF orientation would be of interest. Therefore, droplet evaporation mechanism (Study I) and CNF orientation in the evaporated nanocellulose surfaces (Study I) and nanocomposite films (Study III) were studied. The evaporation dynamics of a c-CNF droplet and a water droplet is remarkably different, as Figures 3 and 4 show. In Study I, image-based analysis was used to confirm the difference in the contact line movement of the evaporating c-CNF and water droplets. During the c-CNF droplet evaporation, a controlled and symmetrical droplet shrinking towards the center was observed, while the shrinking of the deionized water droplet was more asymmetric as shown by severe pinning in random places of the contact line. The controlled, symmetrical shrinking of the c-CNF enables repeatable preparation of aligned c-CNF surfaces for, for example, in vitro cell studies in which several parallel samples are required.

Although evaporated suspensions of both u-CN and c-CN showed a significant amount of anisotropy, only c-CNF was analyzed in more detail due to the interesting cell responses on the dried c-CNF surfaces. On the other hand, u-CN was not further explored in any other studies in this thesis due to the poor cell adhesion in Study I.

Surfaces with aligned c-CNF were smooth and had low surface roughness values and tightly packed fibers, which made the detection of the nanofiber alignment challenging. It also resulted in poor contrast in HIM (Study I) and SEM (Study III) images of the aligned surfaces. In addition, the samples were beam sensitive. However, various techniques – including AFM, HIM, OM, SEM and orientation analyses used in Study I and Study III to detect orientation – all indicate c-CNF alignment. To our knowledge, Study I of this thesis was the first report to show alignment of c-CNF or any CNF along the evaporating droplet boundary line. Recently, Mariani et al. (Mariani et al. 2019) reported CNF alignment observations
consistent with our results presented in Study I. In their study, the contact line moves as the printed line dries and CNFs are deposited on the substrate, similar to those obtained with contact printing in Study I.

Next, the proposed alignment mechanism will be discussed in more detail. In Studies I and III, the presented alignment mechanism is based on the theory introduced by Mashkour et al. (Mashkour et al. 2013) for CNCs. It is notable that CNFs are entangled webs of fibrils, and one cannot easily extend Mashkour’s theory to a CNF suspension upon drying. In our studies, however, we observed CNF alignment in low concentration (0.15w%) in which CNFs do not form entangled web of fibrils and are more free to move relative to each other in suspension. Suggested alignment mechanism of CNF during evaporation is optimized for this particular concentration and will not likely apply for CNF suspensions with higher concentrations. Mashkour et al. (Mashkour et al. 2013) suggested that capillary force gradient and surface tension torque (STT) near a dry-line boundary layer are mainly responsible for CNC alignment. The dry-line boundary layer is the air-suspension-substrate interface, which is later referred as a triple line. Here, CNFs are used as an example instead of CNC. Surface tension torque refers here to a surface-tension-induced rotating moment that can affect orientation of the CNFs in aqueous suspensions close to the triple line and align them parallel to this line. The CNF alignment takes place very close to the triple line. The free energy of a CNF on this interface is different from that on the suspension phase. Whether it is lower or higher depends on the interaction of the CNF with the suspending liquid, underlying substrate, and air. When CNF is on the triple line, the area of suspension liquid exposed to air is reduced. This reduces the surface free energy of the suspending liquid and favors the CNF to be on the interface. It is assumed that the contact of a CNF with this interface (triple line) is energetically favorable. The most significant forces that control the alignment and movement of CNF are Brownian motion of water molecules during evaporation and the surface tension. Brownian motion induces movement of colloidal CNFs and allows them to rotate and translate. If the Brownian motion of water molecules induces CNF approaching the triple line, one end of the fibril may eventually contact the triple line. When the contact line is energetically more favorable than the suspending liquid, the surface tension torque acts on the fibril and rotates the CNF toward the contact line. This induces other CNFs from the suspension to accumulate – upon evaporation of the suspending liquid – onto the CNFs already located on the triple line (Mashkour et al. 2013). The right-hand side of Figure 19 describes this schematically. Generally, the magnitude of a torque (τ) is:
\[ \tau = lF \sin \theta \]  
(2)
in which \( l \) is the length of the lever arm vector, \( F \) is the magnitude of the force, and \( \theta \) is the angle between the force vector and the lever arm vector. When \( F \) is the surface tension force, its magnitude is:

\[ F = l \sigma \]  
(3)
in which \( \sigma \) is surface tension and \( l \) is the length of CNF on which surface tension works (Mashkour et al. 2013).

CNFs close to the triple line are affected by the STT which is a function of the two angles \( \alpha \) and \( \beta \) (Figure 19). Angle \( \alpha \) is the contact angle and angle \( \beta \) is between the long axis of the affected CNF and the triple line. When one end of CNF contacts the substrate surface, and the CNF is very close to the triple line, a capillary force gradient is believed to form between the substrate surface and the CNF. The maximum capillary force is close to the contact point of the CNF and the substrate surface, and the capillary force gradually decreases to the other end of the CNF. The CNF is affected by the Brownian motion of water molecules, and the existence of this gradient turns the CNF parallel to the triple line to minimize the capillary gradient. Once the \( \beta \) angle decreases, the capillary gradient decreases to the direction of increasing capillary forces. When the \( \beta \) angle decreases, the effective surface tension force enlarges. This acts on the CNF and pulls it on the substrate surface, aligning it parallel to the evaporating boundary line (Mashkour et al. 2013). The substrate surface and the CNF surface have different surface energies, so these two contact angles likely differ. This is described using different subscripts in \( F_s \), \( F_{sl} \) and \( F_l \). The contributions of the decreasing \( \beta \) and \( \alpha \) angles on the effective surface tension force are given in Equations 4 and 5 as follows:

\[ F_s = F_{sl} + F_l \sin \left( \frac{\pi}{2} - \alpha \right) \]  
(4)

where \( F_s \) is the surface tension force of the substrate, \( F_l \) is the surface tension force of the liquid, \( F_{sl} \) is the interfacial surface tension force, and \( \alpha \) is the contact angle of the CNF suspension on the PDMS substrate, as described in Figure 19.

The magnitude of STT that affects CNF (according to Equation 2) can be determined from:

\[ \tau_s = lF_s \sin \left( \frac{\pi}{2} - \beta \right) \]  
(5)
in which \( F_s \) is the magnitude of the surface tension force of the substrate, \( \beta \) is the angle between the lever arm vector and the Force vector, and \( l \) is the length of CNF meaning that longer CNFs are subject to the higher STT (Mashkour et al. 2013).
In summary, the forces that act on the fibril include the surface tension gradient and the forces induced by surface evaporation (including Brownian motion of water molecules, shear force and capillary force), which are the driving phenomena behind the alignment of CNFs. The resultant surface tension torque, proportional to the length of CNFs and the horizontal contact angle between CNFs and the surface of the substrate (PVAm-coated glass cover slips in Study I and polydimethylsiloxane, PDMS, in Study III) rotates the CNFs and aligns them parallel to the triple line, similar to how it was described by Mashkour et al. (Mashkour et al. 2013) in the case of CNCs. The difference in our case is, that we do not have an inclined substrate surface, and the length and the surface chemistry of the aligning nanoparticle differs from Mashkour et al.’s study.

In Study I, only c-CNF were included in the suspension, and their alignment is assumed to follow the aforementioned theory. In Study III, the suspension includes c-CNF and c-CNF/MWCNT dispersion and it is the added c-CNF in the suspension that is responsible for the alignment. According to NMR results (Section 5.3), the hes-c-CNF/MWCNT cluster has a strong interaction between the hes-c-CNF and MWCNT components. Therefore, no individual MWCNTs are expected to align because they form a cluster with c-CNF.

Figure 19. The left-hand side shows the shrinking droplet from the side on top of a PDMS substrate in Study III and the right-hand side shows the droplet from above. First, induced by evaporation and Brownian motion of water molecules, one fibril end (green c-CNF fibril on the right-hand side) comes into contact with the triple line. When the β angle decreases, there is increasing effective surface tension force of the substrate ($F_s$) that acts on the CNF. This force pulls the CNF on the substrate surface and aligns it parallel to the dry-line direction. This is the case when the contact of a CNF with the triple line is energetically favorable. This means that when CNF is on the triple line, the area of suspending dH$_2$O exposed to air is reduced, which reduces the surface free energy of the liquid and favors the CNF to be on the interface. $F_l$ is the surface tension force of the liquid and $F_{sl}$ is the interfacial surface tension force.
The interactions that are typically involved in the self-assembly process occurring at colloidal, molecular, or atomic length scale are mainly non-covalent, including van der Waals forces, hydrophobic, electrostatic, hydrogen bonding, π–π aromatic stacking, etc. These are normally weak interactions compared to covalent linkages, but when present in high numbers, they may form extremely stable self-assembled structures. Weak and noncovalent interactions also play a notable role in the alignment of nanoparticles in ordered structures and are therefore the main forces that facilitate self-assembly of the nanoparticles (Yadav, Sharma, and Kumar 2020). Evaporation of the solvent enriches the concentration inducing noncovalent weak interactions and self-assembly. In such process, self-assembly follows a balance of entropy arguments and weak interactions between the assembling chains (Wei, Bai, and Fan 2019). In the case of c-CNФ, the capillary forces are strengthened by cationic charges. Subsequently, the adjacent fibers are pulled closer to each other during drying. In addition, particle concentration, length, surface charge, pH, salt concentrations, and temperature (Zhao et al. 2015) substrate properties, such as roughness, wetting, and surface tension, are likely to affect alignment during evaporation. Therefore, in order to understand the mechanism controlling the alignment of CNФs during droplet evaporation, wider investigation is still required. The self-orienting capacity of CNФs is not a well-described phenomenon.

When comparing the results of different alignment studies with different cellulose nanomaterials, it is necessary to consider differences in nanocellulose origin, extraction techniques, modifications, and experimental setups. A controlled assembly of nanomaterials into aligned patterns requires a thorough understanding of the drying process of the nanoparticle solution/suspension and controlled manipulation of the shape of the evaporating dry-line boundary (Beyer and Walus 2012; Wei and Zhiqun 2012). The shape of the free evaporating boundary line can potentially be adjusted using appropriate substrate, and optimal surface energies of the suspension, as well as that of the substrate.

Mashkour et al. (Mashkour et al. 2013) suggested that STT has a negative effect on the alignment of CNC under magnetic and electric fields. Therefore, a considerable amount of energy would be required to align CNCs under these fields, to overcome the STT when a liquid suspension evaporates to form the final film. In addition, the final alignment would often be unsatisfactory. Similar challenges are expected to arise during active CNФ alignment due to simultaneous forces acting on the self-aligning of CNФs. This was one reason why the self-assembly method was selected in this study. Another reason was the requirement of repeatability. Droplet evaporation-induced self-assembly was a suitable and straightforward way to
produce many samples for cell experiments, which often requires many repeat and parallel samples.

6.2 Substrate and nanomaterial properties

6.2.1 Cellulose nanofiber surfaces, coatings and nanomaterials in Studies I and II

The properties of different CNF coatings and surfaces, as well as CNF nanomaterials used in Studies I, II, and III are discussed here. In Study I, u-CNF, a-CNF and c-CNF surfaces were prepared on glass coverslips, while in Study II, CNF-based coatings (a-CNF, c-CNF, and c+a-CNF) on a microfibrous cellulose mesh were used to improve the properties of the mesh as a cell carrier; such as in skin tissue engineering and wound healing applications.

The size of the CNF was smaller in charged c-CNF and a-CNF than in u-CNF (Study I). In general, the higher the fiber charge, the smaller the fibril diameter of a CNF (Pöhler et al. 2010). The surface roughness of the CNF surfaces was measured in Studies I and II. For a-CNF and c-CNF coatings, $R_s$ was slightly higher in Study II than in Study I (in the range of 4.52–5.68 in Study I and 9.04–10.2 nm in Study II), while that of the combination coating of Study II was notably higher, at 55.76 nm. This could be due to strong ionic crosslinking between anionic (-COO⁻) and cationic (-N(CH₃)₃⁺) groups, which might result in local aggregation of the nanofibers and higher roughness of the c+a-CNF coatings.

The dynamic contact angles of CNF coatings used in cell experiments in Studies I and II are consistent in that the a-CNF coatings provide lower contact angles $29.64^\circ$ (Study I) and $31^\circ$ (Study II), while for the c-CNF they were $67.70^\circ$ (Study I) and $65^\circ$ (Study II). In Study I, the more neutral u-CNF coating showed a contact angle $32.56^\circ$. A combination coating c+a-CNF (Study II) had a contact angle of $32^\circ$, similar to that of a-CNF, which was an interesting finding. Combination coatings were fabricated so that a-CNF was mixed with c-CNF “inside” the c-CNF droplet. This was necessary, as an a-CNF droplet on a cellulose mesh was absorbed by the mesh, while c-CNF retained its droplet shape after application on top of the cellulose mesh.

In Study II, the variation of the volume (600 µL or 150 µL) of the CNF suspension and the used CNF type created two different coating micro-
topographies: flat 2D film-like coatings on the surface of the cellulose mesh and 3D coatings that covered the individual mesh fibers and filled the pores between the fibers. Film-like coatings were predominantly formed from c-CNFS, while a-CNFS leaked into the mesh pores, likely due to smaller and more homogeneous fiber size presented in Study I. As shown in Figures 2 and 3 in Study I, c-CNFS contains also larger fibers. The larger fibers of c-CNFS suspension and a smaller number of CNFS binding sites (hydrogen bonding sites) likely caused the film-like coating, while the smaller a-CNFS and the higher number of hydrogen bonding sites allowed its penetration deeper into the mesh pores and stronger interaction with cellulose mesh, respectively.

The film forming property was utilized for the preparation of c+a-CNFS coatings; the c-CNFS applied first stayed as a droplet on top of the mesh and enabled subsequent a-CNFS mixing “inside” the droplet. This resulted in gel-like coating due to fast ionic crosslinking between the oppositely charged sidechains of a-CNFS and c-CNFS as the trimethylammonium (−N(CH3)3+) group of c-CNFS can form ionic bonding with carboxyl (−COO−) groups of a-CNFS. (Montazer and Harifi 2018) The larger c-CNFS fibers blocked the pores, which – in addition to crosslinking – prevented the penetration of a-CNFS.

The differences in chemistry and size of the functional groups of CNFSs in different coatings affects the wettability and the water uptake, which resulted in differences in swelling properties and softening dynamics of the coatings. The more hydrophilic surface of a-CNFSs has (−COO−) and (−OH) functional groups that enable hydrogen bonding (Chaker and Boufi 2015), and therefore bind more water on the surface, resulting in a lower contact angle than the more hydrophobic c-CNFSs, which contains (−OH) groups and more hydrophobic methyl (−CH3) groups. The latter does not form hydrogen bonds (Chaker and Boufi 2015). The hydrogen-bonding capacity may also influence the penetration of the a-CNFS fibers into the mesh pores, as the cellulose mesh has more hydrogen-bonding sites for (−COO−) and (−OH) groups of a-CNFSs than binding sites for the (−CH3) of the c-CNFSs (Chaker and Boufi 2015). However, the c-CNFS has a larger functionalized moiety than a-CNFS, so it forms a more branched and spongy structure, resulting in higher water uptake (K. Zhang et al. 2019) than a-CNFSs. The positively charged (−N(CH3)3+) groups interact with dipolar substituents (Headley and McMurry 1994), enabling solvation even though the (−CH3) groups make the c-CNFSs more hydrophobic. This leads to higher swelling ratio and lower surface stiffness of c-CNFS coatings.
Swelling over time was higher for a-CNFC coatings than c-CNF coatings. The negative surface charge combined to hydrogen bonding with water molecules might affect observed swelling and softening of a-CNF coatings over time. The branched sponge-like structure of $(−\mathrm{N(CH}_3)_3^+)$ groups could affect the higher total water uptake of c-CNF coatings. The swelling of c-CNFCs decreased slightly and, correspondingly, the mean stiffness increased slightly over time, especially in DMEM. This may be due to the ions in DMEM, which may enable ionic cross-linking, resulting in reduced water uptake and increased strength of the material in time. Logically, the stiffness of the measured samples decreases with increased swelling of the coatings. Upon swelling, the material absorbs water and softens. The microtopography of the c+a-CNFC coating is similar to that of c-CNFCs, while the contact angle, swelling, and stiffness properties resemble those measured on the a-CNFC coatings. This can be due to strong ionic cross-linking between a-CNFC and c-CNFC, preventing the water absorption. However, the stiffness of the c+a-CNFC coating did not decrease over time, which could be due to the strong ionic cross-linking, resulting in reduced interactions between hydrogen-bonding groups and water molecules.

The properties of CNFs and nanocellulose products are highly dependent on the chemical modification as well as on the starting materials, including whether CNF is extracted from hardwood or softwood pulp. In this discussion, the focus is mainly on the differences resulting from chemical modification between a-CNFC and c-CNFC. However, it is worth noting that the studied a-CNFC and c-CNFC are extracted from softwood and hardwood cellulose pulps, respectively, and generally there are differences in properties between hardwood and softwood pulps, regarding structural, morphological, thermal and rheological behavior. (B. Li et al. 2018; Sanchez-Salvador et al. 2022) For instance, Li et al. (B. Li et al. 2018) showed that CNF derived from hardwood pulp exhibited larger particle size, higher crystallinity, and higher thermal stability, compared to the corresponding CNF obtained from softwood pulp under the same processing conditions. Differences in crystal size and crystallinity of cellulose results in the differences in morphology of CNFs and their rheological properties. For instance, hardwood with higher content of amorphous cellulose has been reported to be more prone to keep the fibrillar structure, while softwood fibrils broke more easily. (Sanchez-Salvador et al. 2022)
6.2.2 Anisotropic nanocomposite films and nanomaterials in Study III

Nanocomposite films with either isotropic or anisotropic conductivity were fabricated in Study III. The evaporation of hes-c-CNF/MWCNT dispersion resulted in an isotropic film (control), while additional c-CNFs suspended in the dispersion-induced self-assembly during evaporation and resulted in an anisotropic (assembled) film. The alignment observed in Study I is consistent with the results of Study III. Thus, we postulate that the suspended free c-CNFs are responsible for the assembly along the boundary line in assembled films. The alignment mechanism in Studies I and III is assumed to be the same, based on alignment of c-CNF, and was discussed in more detail in Section 6.1.

In Study III, the films were fabricated on top of PDMS substrates. Interaction of c-CNF with its substrate glass (Study I), cellulose (Study II), and PDMS (Study III) is different. As a result, a firm non-detachable c-CNF coating formed on glass in Study I, while detachable free standing nanocomposite films were obtained in Study III. In addition to nanocomposite films, pure c-CNF detachable films were fabricated on top of PDMS substrates (unpublished).

Figure 7 is an oversimplification of the hypothesized structure of the film and the nanocomponents are not to scale. The idea is to show the aligning component, free c-CNF, and the more entangled hes-c-CNF-MWCNT component. Alignment of c-CNF between hes-c-CNF-MWCNT creates the "circular zones". Between the more entangled hes-c-CNF-MWCNTs, there are aligned c-CNFs, and these components alternate along the radial direction creating the "circular zones". The diameter and the length of c-CNFs are, on average 5–15 nm and less than 2 μm, respectively (Pöhler et al. 2010), while those of MWCNTs are, on average approximately 9.5 nm and 1.5 μm, respectively. Sonication treatment during the preparation of the dispersion significantly fibrillates and cuts the length of hes-c-CNF. The dispersion consists of hes-c-CNF-MWCNT clusters in which cationic groups point outwards. Suspended c-CNFs are free to interact with such cationic groups after initiation of the self-assembly at the evaporating boundary line. These free c-CNFs initiate the assembly process as described in Section 6.1, and result in aligned free c-CNFs during droplet evaporation and assembly of the hes-c-CNT-MWCNT entangled component. This forms the structure with alternating the aligned c-CNFs and the more entangled hes-c-CNF-MWCNTs. Electrically conductive composites generally contain both well-conducting and insulating phases, which inevitably results in the presence of gaps, defects, or complete lack of contact among the electrically conductive phase of the composite (H. Zhang et al. 2019). In Study III, this fact was
utilized in fabrication of anisotropic nanocomposite films, as the aligned c-CNF induced these non-conducting gaps (Figure 2). Furthermore, the width and number of insulating c-CNF gaps can be controlled to some extent with the amount of additional c-CNF used. A higher amount of additional c-CNF results in higher resistance, indicating an increase in insulating gaps. This could be due to the increased width of c-CNF insulating gaps, as could be the case between Samples S1 and S2. Suspension S2 has higher concentration of suspended c-CNF than S1; this can end up separating more conducting MWCNT networks, which would result in an increased number of insulating c-CNF gaps. At a larger scale, the anisotropy is detected from the surface topography using image analysis (Section 5.1.3), which also presents that the control films are more isotropic.

Study III shows a remarkable difference in electrical properties along the zone and towards the center. The different electrical properties between the control film and the self-assembled films were shown using resistance measurement. The resistance measurement was also used to investigate the level of anisotropic conductivity in the assembled films, including the different resistance along the radial line and along the circular zone. IR-images and resistance measurements showed substantially different conductivity of the assembled films along the radial direction compared to the circular zone direction. The aligned non-conducting c-CNFs along the circular zone is suggested to be responsible for this conductivity difference between directions. These c-CNFs are packed next to each other along the neighboring circles. Towards the center, more and more CNFs are packed between the conducting MWCNT components, blocking the conductive pathways and increasing resistance towards the center. Observations in Study III indicate a resistive component along the zone and a resistance coupled to a capacitive component along the radial line and nonlinear IV-curves along the radial line of the assembled nanocomposite films.

To study the effect of the size of the nanofibers on the properties of the anisotropic nanocomposite films, free c-CNF was high-energy-sonicated before the preparation of anisotropic nanocomposite film (S3). The concentration of the free c-CNF in S3 was equal to that of S2. Sonication breaks the fibers, and the resulting smaller sized free c-CNFs increased the resistance of film S3 compared to that of film S2 with equal c-CNF concentration. It seems that smaller c-CNFs in S3 reduce conductive pathways more than larger c-CNFs in S2. In other words, the smaller c-CNFs of S3 may cover MWCNTs more, blocking the conductive pathways.

Free c-CNF alignment is expected to take place along the evaporating boundary line, and a such film edge is expected to be relatively smooth, as was observed in
Study III. The smooth film edge of the self-assembled film is slightly thickened in bottom right of Figure 10. This is consistent with detected droplet boundary line pinning in the beginning of evaporation in Study I, which results in an accumulation of aligned fibers on the boundary edge. Therefore, a slight ‘coffee ring’ effect is expected in the beginning of the evaporation. Subsequent even progress of the evaporation results in alignment of the c-CNPs parallel to the boundary line. Faster evaporation closer to the film center likely affects the reduced anisotropy in the center parts of the films (Study III) and surfaces (Study I).

According to NMR study, the trimethyl protons of the cationic substituent remain unchanged in all samples. Therefore, cationic functional groups are assumed to point outward from MWCNTs, while the less solvated area refers to cellulose ring protons (H3–H5), which would be the location of the stronger interaction with MWCNTs. The NMR result is in accordance with hypothesis (Figure 8f) and (S)TEM (Figure 6h of Study III) in which hes-c-CNPs is covering MWCNTs. This result also provides valuable information regarding the orientation of c-CNPs relative to MWCNTs. Such a structure, with cationic functional groups pointing outwards from the hybrid structure, could result in a stable dispersion and prevent aggregation of MWCNTs. According to NMR and ATR-FTIR result of Study III, sonication treatment does not result in covalent chemical changes in the dispersion components. SEM studies of Study III show dispersed MWCNTs without self-aggregates. This is consistent with the NMR finding, which indicated robust connections between c-CNPs and MWCNT at the molecular level. Therefore, c-CNPs can be used to disperse MWCNTs; however, its dispersion effect should be further evaluated.

The main novelty in Study III was the introduced nanocellulose-based material showing anisotropic conductivity. To our knowledge, it was the first report to show dispersion of MWCNT with c-CNPs, and formation of CNF-based nanocomposite films with anisotropic conductivity. The material’s conductivity is up to 1000 times higher along the dry-line boundary than along the radial direction. The material and the method to produce it is novel, in which the alignment of c-CNPs along evaporating the dry boundary line is used. In this, c-CNPs has a role to disperse MWCNTs, and as an additional suspension element the other function of c-CNPs is to create the desired anisotropy. This has not been utilized before. When nanocomposite film is manufactured using the high-energy-sonicated c-CNPs/MWCNT dispersion without suspended c-CNPs, evenly conducting nanocomposite films are obtained. Therefore, we suggest that suspending additional
c-CNFs in the c-CNF/MWCNT dispersion results in nanocomposite films with anisotropic conductivity.

6.3 Cell responses to cellulose nanofiber surfaces

Remarkable elongated morphology of MEFs was observed and repeatable in Study I on aligned c-CNF surfaces. To our knowledge, Study I of this thesis was the first report to show intentional cell alignment along aligned c-CNF surfaces.

The dynamic contact angles of the u-CNF, a-CNF and c-CNF surfaces used for the cell experiments in Study I were 32.56°, 29.64°, and 67.70°, respectively. The significantly higher contact angle value on the c-CNF surface may have an effect on the adhesion and spreading of the cells, but this was not studied in Study I. In Study II, the contact angles of a-CNF and c-CNF were similar to those of Study I. The adhesion sites of the cell (focal adhesions) are 5–200 nm. The repeatable size of the aligned topography in Study I was in this size range and might have an enhancing effect on cell orientation along the aligned c-CNF. The surface charges, chemical functionalities, fiber dimensions, morphology, and CNF arrangement probably all influence on the cell adhesion, proliferation, and survival.

Engineering artificial skin constructs is an ongoing challenge and the ideal material for hosting the skin cells is still to be discovered. This was a motivation for Study II. A promising candidate for a skin cell carrier is the low-cost cellulose that is commonly fabricated in the form of mesh and is applied as a wound dressing. However, the structure and topography of current cellulose meshes are not optimal for cell growth, which was the reason for applying the CNF coatings. The non-coated meshes in Study II had high porosity with large voids, which alone can affect poor cell adhesion upon plating on non-coated meshes. However, SEM images of the rare cells adhered to the non-coated mesh confirm poor cell survival, as the morphology of the cells is round with apoptotic vesicles visible on the cell surface, indicating that the cells have started the process of programmed cell death. Therefore, in Study II, the charged CNFs were used as biocompatible coatings of cellulose meshes for improved cell adhesion.

Study II concluded that the flat 2D substrates of c600, a600, and c+a could be suitable for cell sheet technology. In the 3D substrates of c150 and a150, the cells penetrated into the material and could be used in tissue engineering research. It is possible to modulate the cell-material interactions by the elasticity, roughness, and surface chemistry of the material. Natural tissue elasticity varies from approximately
1 kPa for soft tissues including brain and fat, to 10–30 kPa for medium-soft tissues including skin, to hard tissues (> 105 kPa) including bone (Skardal et al. 2013; Yang et al. 2018). The stiffness of studied CNF coatings is similar to the those of fibrotic tissue (100 kPa), cartilage and tendon (>100 kPa) (Discher et al. 2005; Skardal et al. 2013). Compared to conventional cell culture substrates with stiffness at the GPa-level, the CNF substrates used in Study II were notably soft. However, at the cell perception level, the used CNF substrates are relatively stiff. The studied CNF surfaces differ in their microtopography, roughness, swelling ratio, and Young’s modulus, and especially in surface chemistry. The latter was suggested to have the major role in cell adhesion, as it affects the composition and speed of protein adsorption on the CNF surfaces (Syverud 2017). In Study II, greater protein adsorption was observed on c-CNPs surfaces, compared to a-CNPs surfaces, a result consistent with previous studies (Attwood et al. 2019; Courtenay et al. 2019). However, the protein composition is more important than the number of adsorbed proteins (Hoshiba et al. 2018), and it has been shown that (−COO−) groups on the most superficial layer of the substrate in general have a positive effect on cell adhesion, spreading, and proliferation (Courtenay et al. 2017; Faucheux et al. 2004; Hoshiba et al. 2018). Bovine serum albumin (BSA) adsorption on c-CNPs coated meshes likely reduced the amount of adhered NHDFs, while the attachment of the ADSCs was not affected (Hoshiba et al. 2018). Based on previous studies (Courtenay, Deneke, et al. 2018; Courtenay et al. 2017) and consistent similar results in Study II, it was suggested that the ADSCs experience integrin-independent charge-mediated adhesion on positively charged c-CNPs surfaces due to very fast adhesion. Fast adhesion on c-CNPs surfaces was also observed in serum-containing medium in Study II, indicating fast adhesion before the adsorption of BSA. Similar unpublished observation was done in Study I, in which MEFs adhered and begin to align on c-CNPs surfaces soon after plating, in a serum-containing medium. After fast adhesion of ADSCs on c-CNPs coated meshes, their proliferation was suppressed and during time, clusters, and spheroids were formed.

Both a-CNPs and c+a-CNPs coatings were suitable for cell adhesion and growth. The level of adsorbed BSA on c+a-CNPs was comparable to the level of less hydrophilic c-CNPs. However, the hydrophilic surface of c+a-CNPs likely enhanced replacement of BSA by the cell adhesion-mediating proteins in the most superficial layer of the adsorbed proteins and maintained cells viable longer. The c+a-CNPs construct proved to combine the benefits from both types of CNF, which means that the c+a-CNPs thin film cell carrier is feasible for further investigation in skin tissue engineering. Even though the c-CNPs coatings did not support long-term cell-
growth, they might have other roles in tissue engineering, such as the formation of 3D multicellular spheroids or an alignment initiator in the case of aligned c-CNF surfaces obtained in Study I.

To our knowledge, Study II of this thesis was the first report to show coculture study of ADSC and NHDF on c-CNF-coated surface with remarkably different adhesion on the surface. According to the coculture experiments of Study II, NHDF did not properly adhere and begin to spread on the c-CNF surface, while ADSC adhesion and spreading was rapid. As a summary, CNF coatings, even when using the same CNF type can have diverse cell responses depending on the cell type, coating preparation and degree of anisotropy. It has been shown that fast adhesion, even with serum-free medium was achieved for ADSC, while anisotropic c-CNF promoted cell elongation along the aligned c-CNF in Study I. The negatively charged a-CNF coating remarkably improved the proliferation of both cell types. In Study II, the positively charged c-CNF coating only significantly enhanced adhesion of ADSCs. The c+a-CNF construct combined the benefits from both types of CNF, which means that the c+a-CNF thin film cell carrier is feasible for further investigation.

6.4 Future perspectives

Although progress has been made in CNF alignment and subsequent controllable cell alignment, well-defined 3D structures with alignment and other tailored properties remain challenging. In addition, CNF alignment and interactions require in-depth understanding in order to develop more controlled aligned self-assembled structures. While this thesis has presented c-CNF alignment, many applications would benefit from the alignment of CNF grades with negatively charged functional groups. Detailed understanding on the cell adhesion and alignment, as well as cell-type-dependent differences on current surfaces, would benefit future work. Controlled cell growth guidance would enable the development of tissue mimicking in vitro models. These would reduce the need for animal testing and enable more accurate testing of, for example, new drug effects on specified cell types in native tissue mimicking environment. In addition, despite intensive studies, charge-mediated adhesion remains poorly understood. Better understanding could aid in the development of applications that take advantage of different cell responses on c-CNF observed in the present study.
As shown in Study II, the combination coating provides the beneficial properties of both a-CNF and c-CNF and lacks the disadvantageous properties of c-CNF; namely, poor adhesion of NHDF and detachment of NHDF and ADSC after a period of cultivation. The combination coating c+a-CNF could be studied further for its potential use in biomedical engineering in different applications.

Anisotropic materials are important functional materials in many fields. Study III shows a remarkable difference in electrical properties along the circular zone and towards the center. Observations in Study III indicate a resistive component along the zone and a resistance coupled to a capacitive component along the radial line and nonlinear IV-curves along the radial line of the assembled nanocomposite films. Deeper understanding of the assembled films of Study III, would enhance the use and further development of the discovered method to produce nanocellulose-based material showing anisotropic conductivity.

One challenge encountered in Study I and III was the lack of characterization method of aligning c-CNFs during droplet evaporation. In addition, it was not straightforward to characterize surfaces with aligned CNFs. Therefore, there is a need for improved techniques for nanoparticle alignment characterization or nanoparticle tracking in suspension, especially during droplet evaporation.

The current work provides a method to produce surfaces with aligned c-CNF and aligned electrical properties, which can be developed further with same materials or using novel materials. Furthermore, the current technique can be used to produce surfaces with aligned c-CNFs to investigate the effect of alignment in different applications, or with varying cell lines. Surfaces with aligned c-CNF could be adjusted by adsorbing negatively charged molecules with an attempt to retain the aligned topography.
The aims of this dissertation were (i) to produce anisotropic cellulose nanofiber surfaces to guide cell orientation, and (ii) to develop nanocomposite substrate with anisotropic thermal and electrical conductivity. Based on the results of Studies I–III, the following conclusions can be drawn:

1. The evaporation-induced self-assembly technique was used to align CNF along the evaporating droplet boundary line.
   - Aligned anisotropic c-CNF surfaces were produced (Study I)
   - CNF-driven assembly was used to produce nanocomposite films with anisotropic conductivity. (Study III)
2. Anionic and cationic CNF surfaces promoted cell growth and/or adhesion in these studies, while the unmodified CNF surface did not support cell growth.
   - Mouse embryonal fibroblast proliferation and viability was the highest on a-CN F surfaces, but also c-CN F surfaces promoted cell proliferation. (Study I)
   - Human adipose-derived stem cell growth was highest on a-CN F-coated cellulose meshes. c-CN F-coated cellulose meshes induced fast adhesion of ADSCs. Their viability on c-CN F-coated meshes after the first day was not comparable to that of ADSCs on a-CN F and c+a-CN F. (Study II)
   - Human dermal fibroblast grew well on a-CN F coated and c+a-CN F coated meshes. Their viability on c-CN F coated meshes were poor, although better than on uncoated cellulose meshes (Study II)
3. Mouse embryonal fibroblasts were shown to orient and elongate along aligned CNFs.
   - When analyzed using orientation analysis, the morphology of MEF was significantly more anisotropic than that of MEFs grown on a glass substrate (Study I)
4. Charged CNF can be used to control cell growth and other nanomaterials
   - Cell adhesion varies depending on the cell line and functionalization (Studies I–II)
• Alignment of CNF was achieved using c-CNFS (Study I), which further resulted in alignment of cells along the aligned c-CNFS (Study I).
• Finally, alignment of c-CNFS along the evaporating boundary line resulted in the formation of anisotropic electrically conductive composite film (Study III). Potentially, other substances can be controlled using alignment of c-CNFS along the evaporating boundary line.

5. It was possible to obtain nanocomposite films either with isotropic or anisotropic electrical properties. Isotropic films were obtained when hes-c-CNFS/MWCNT dispersion was evaporated. Anisotropic films were formed when additional CNF was added to the dispersion-inducing CNF alignment along the evaporating boundary line (Study III).
• Evaporation of hes-c-CNFS/MWCNT dispersion resulted in evenly conducting isotropic nanocomposite films.
• Mixing of additional c-CNFS with hes-c-CNFS/MWCNT dispersion forms a suspension. The evaporation of this suspension resulted in nanocomposite films with anisotropic conductivity. The material has up to 1000 times higher conductivity along the dry-line boundary direction than along the radial direction. The anisotropic film also had anisotropic thermal conductivity.

The main aim of the dissertation was to develop self-assembly methods for creating aligned and functional CNFs and composite film surfaces. The aim of Study I was to develop surfaces with aligned CNFs in order to orient cells along the aligned CNFs. Evaporation-induced self-assembly was used to produce surfaces with aligned CNFs for the first time, which also promoted cell orientation along the CNF alignment direction. The aim of Study III was to develop a method to align c-CNFS in the presence of a MWCNT component and obtain electrically anisotropic nanocomposite films. The CNF-driven self-assembly method used in Study I was used in Study III for the first time to manufacture anisotropic electrically conductive c-CNFS/MWCNT nanocomposite films.

The aim of Study II was to improve cytocompatibility of a low-cost cellulose mesh using charged CNF coatings. Studied a-CNFS and c+a-CNFS coatings were shown to support cell growth and thus improve the cytocompatibility of a cellulose mesh. To our knowledge, Study II was the first to show a coculture study of ADSC and NHDF combination on c-CNFS with remarkably different adhesion on the
surface. ADCS adhesion and spreading on c-CN coating was rapid, while only poor adhesion and spreading was detected for NHDF. As a summary, CNF coatings, even when using the same CNF type, can have diverse cell responses depending on the cell type, coating preparation, and degree of anisotropy.
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Cellulose Nanofiber Alignment Using Evaporation-Induced Droplet-Casting, and Cell Alignment on Aligned Nanocellulose Surfaces

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ABSTRACT: This work investigates droplet-evaporated cellulose nano fiber (CNF) alignment and cell responses on CNF surfaces. Surfaces of unmodified (u-), anionic (a-), and cationic (c-) CNFs were fabricated using an evaporation-induced droplet-casting method and characterized in terms of degree of orientation. Circular variance (CV) values obtained using Cytospectre software to analyze the degree of orientation from AFM images showed a significantly higher degree of orientation on c- and u-CNf surfaces (average CV 0.27 and 0.24, respectively) compared to a-CNf surfaces (average CV 0.76). Quantitative analysis of surface roughness plots obtained from AFM images confirmed the difference between the direction of alignment versus the direction perpendicular to alignment. AFM images as well as observations during droplet evaporation indicated c-CNf alignment parallel to a dry-boundary line during droplet evaporation. Fibroblasts were cultured on the u-, a-, and c-CNf surfaces with or without a fibronectin (FN) coating for 48 h, and the cell response was evaluated in terms of cell viability, proliferation, morphology, and degree of orientation. Cell viability and proliferation were comparable to that on a control surface on the a-CNf and c-CNf surfaces. Although an FN coating slightly enhanced cell growth on the studied surfaces, uncoated a-CNf and c-CNf surfaces were able to support cell growth as well. The results showed cell orientation on aligned c-CNf surfaces, a finding that could be further utilized when guiding the growth of cells. We also showed that the alignment direction of c-CNfs and thus the cell orientation direction can be controlled with a contact-dispensing technique.

1. INTRODUCTION

An anisotropic architecture in most native extracellular matrices (ECMs) of tissues or organs is important for tissue function.1,2 An organized structure that mimics the topographical structures provided by fibrous components of ECMs is therefore believed to be crucial in order to mimic as closely as possible the native ECM for guiding cell growth or tissue regeneration.1,3 Artificial organized structures can be used, for example, in the improved repair process of neural damages, since the structures act as cell guidance structures leading to improved cellular differentiation and axonal reconstruction.5,6 Although a three-dimensional scaffold is often required for the regeneration of tissues, a single cell layer with aligned structures can initiate the development of an organized structure of cell layers and tissues, such as cornea, vascular media, and dermis.7 It is also well-known that surface topography influences cell behavior.8−10 It can strongly influence the polarity of cells through a process known as contact guidance.10−12 Multiple cell types elongate and align parallel to artificial nanogrooves and nanoridges.13−19 Actin and other cytoskeletal elements organize in an orientation parallel to the artificial structures, resulting in the elongation and alignment of the cell.20 Methods of producing aligning channels, grooves, and surfaces include deep reactive ion etching,21 electron beam lithography,22,23 and direct laser writing,24 using a femtosecond laser,25 photolithography,26 plasma dry etching,27 dual etching and bioprinting,28 and electrospinning.2 Electrospinning is used to

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Supporting Information
align fibrous structures, which are of significant interest for tissue-engineering purposes. \textsuperscript{1,29–32} Aligned nanofibrous structures have shown superior capacity in shaping cell morphology, guiding cell migration, and affecting cell differentiation when compared to other types of structures both in vitro and in vivo.\textsuperscript{1,32–34} Cells, their cytoskeletons, and their nuclei have been shown to align and elongate parallel to the fiber axes, when cultured on aligned fibers.\textsuperscript{35} The challenge is to create an aligned porous 3D structure of fibers with a diameter identical to that of native ECM fibers (a diameter less than 100 nm, preferably in the range of 10–50 nm).\textsuperscript{1,2,30,37} Currently, a technically effortless and energy efficient technique for creating aligned substrate layers for the initiation of organized structure development is missing.\textsuperscript{38,39}

Cellulose, the most abundant polymer on earth, is renewable, biodegradable, and nontoxic,\textsuperscript{40} as it consists of anhydroglucose units. The mechanical disintegration of wood fibers produces cellulose nanofibers (CNFs) with a length in the micrometer range and a width in the nanometer range (10–100 nm).\textsuperscript{30,42} Amorphous and crystalline cellulose regions can be separated via acid hydrolysis that degrades the amorphous regions, yielding cellulose nanocrystals (CNCs).\textsuperscript{41} In addition to plant resources, some bacteria produce nanocellulose, referred to as bacterial nanocellulose (BC).\textsuperscript{43}

An increasing demand for renewable and sustainable resources favors the utilization of plant cellulose,\textsuperscript{40} and the advantage of CNFs is that they can be processed at an industrial scale at a relatively low cost,\textsuperscript{42,43} and produced with a variety of functional groups and using several industrially attractive processes.\textsuperscript{44} Several reviews report on the utilization of plant-derived CNFs in composites, as a paper and paperboard additive, and in barrier coatings, food, transparent films, aerogels, absorbents, and biomedical applications.\textsuperscript{45–48} In addition, CNFs are an interesting biological fibrous material for cell culturing applications because of their abundance, their nonanimal biological origin, several chemical modification and functionalization possibilities through the hydroxyl groups on the sugar moieties, their ECM-mimicking fiber dimensions, the possibility for controlled enzymatic degradation, and the possibility of forming aligned topographies.\textsuperscript{41,42,49–53} Other unique properties of CNFs include their crystallinity, rheological properties, barrier properties, low density, mechanical strength, and mechanical reinforcement capability.\textsuperscript{41,42} Chemical modification can improve the processability and performance of CNFs,\textsuperscript{54} for example, by enabling the dispersion of CNFs in most nonpolar polymer matrices,\textsuperscript{55} and can bring advantageous biological properties, such as a resemblance to natural ECM or the addition of bioactive agents on the backbone.\textsuperscript{55} Additionally, chemically added charged groups have an effect on the physicochemical properties of CNFs.\textsuperscript{52}

Although plant-derived CNFs have been extensively studied for a wide range of applications, there are few investigations of their interaction with biological systems, compared to the corresponding studies of the utilization of BC. The biocompatibility of plant-derived CNFs has been demonstrated in many studies\textsuperscript{49,54–59} The surface modification of nanocellulose has been shown to have a direct effect on the biological responses of living cells in contact with nanocellulose substrates. Human dermal fibroblasts showed a measurable effect on their proliferation and morphology when the effects of changes in nanocellulose surface topography, chemistry, and charge were studied.\textsuperscript{56} Hua et al.\textsuperscript{57} have shown an improved cytocompatibility of 2,3-epoxypropyltrimethoxysilane chloride (EPT- MAC)-modified cationic CNFs. Hua et al.\textsuperscript{56} already suggested that CNF fiber alignment might have an advantageous effect on cell adhesion and spreading. They observed fiber alignment with anionically charged CNF films that had been oxidized with 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), prepared by vacuum filtration. They controlled the number of negatively charged groups and found that increased surface charge resulted in increased unidirectional fiber alignment.\textsuperscript{58} In addition, He et al.\textsuperscript{59} have observed cellular alignment on an aligned nanofiber scaffold fabricated with electrospinning.

Aligning nanoparticles with the aim of obtaining unique properties and functions is an interesting research area in the field of designing and creating advanced materials for biomedical applications.\textsuperscript{5,61,62} Significant efforts have been made to obtain aligned, ECM-mimicking nanofibrous structures for cell culturing substrates.\textsuperscript{1} As mentioned above, cellulose nanofibers are unique and promising natural fibers with several remarkable properties; their properties can be enhanced by aligning the CNFs,\textsuperscript{51,52} thus, achieving the reinforcement of composite materials or guiding cell growth with a controlled fiber orientation.\textsuperscript{63} Compared to CNCs, the alignment of CNFs is limited due to their physical dimensions, their microscale length being the most important limiting factor.\textsuperscript{64} To date, a number of methods to align CNFs, including shearing salt- or acid-induced CNF gels,\textsuperscript{51,65} cold drawing,\textsuperscript{66} shear-convective assembly,\textsuperscript{67} electrospinning,\textsuperscript{68,69} wet spinning,\textsuperscript{70} wet stretching,\textsuperscript{71,72} and applying magnetic\textsuperscript{73} or AC electric fields,\textsuperscript{74} have been used.

It is a known phenomenon that nanoparticle droplet drying may result in the formation of ordered patterns on the substrate, depending on the mode of solvent evaporation, which is described in more detail elsewhere.\textsuperscript{61} A droplet casting method has been successfully used for the self-assembly of polymers, proteins, graphene, and nanoparticles such as carbon nanotubes and metal oxides.\textsuperscript{75} It has been suggested that self-alignment behavior depends on the nanoparticle charge and length, the colloidal stability of the dispersion, and the evaporation temperature. In addition, the degree of the alignment may be controlled by changing the ionic strength and pH of the solvent,\textsuperscript{52,65,75,76} Surface tension torque\textsuperscript{77} and evaporation-driven self-assembly\textsuperscript{78} have been shown to control the alignment of CNCs but a review of the literature revealed that there is no report on the self-orientational capacity of CNFs induced by the surface tension torque, evaporation-driven self-assembly, or orientation of charged CNFs in an evaporating droplet meniscus. Here, we present a strategy to produce aligned CNF structures by using the evaporation-induced droplet-casting method. The method described in this paper is simple, quick, efficient, and safe, compared to the above-mentioned methods used to align CNFs, which require more time, high energy consumption, a high level of technical expertise, and, often times, expensive proprietary technology.\textsuperscript{77} A further aim is to investigate the interaction of cells and CNF surfaces possessing different properties, such as different charges and different surface topographies, as well as different surface chemical groups linked to the backbone of the sugar molecule. The cell growth, morphology, and degree of orientation are investigated on unmodified (−)-, anionic (−), and cationic (−) CNF surfaces, and on aligned versus unaligned surface areas.

2. MATERIALS AND METHODS

2.1. Chemicals and Biomolecules

The following biochemicals were purchased from VWR (Helsinki, Finland) and used as received or reconstituted according to the manufacturer’s instructions: Dulbecco’s...
Modified Eagle Medium (DMEM) high glucose, w/o L-glutamine, w/o sodium pyruvate (Biowest, L0101); Dulbecco’s phosphate buffered saline (PBS) w/o calcium, w/o magnesium (Biowest, L0615); trypsin-EDTA 1x w/o calcium, w/o magnesium, w/phenol red (Biowest, L0930); L-glutamate 100X, 200 mM (Biowest, X0550); American fetal bovine serum (Biowest S1520); penicillin-streptomycin solution 100X (Biowest, L0022); AlamarBlue cell viability reagent (G-Biosciences, CAS No. 62758-13-8); and dimethyl sulfoxide (DMSO; AppliChem, CAS No. 67-68-5). Human fibronectin was received from the University of Tampere (The Protein Dynamics Group, Prof. Hytönen), which was purified in house using immobilized gelatin affinity chromatography.

2.2. Preparation of Materials. 2.2.1. Cellulose Nanomaterials. Bleached and never-dried cellulose kraft pulps were used for the production of CNFs. Three different CNF grades were produced: native (u-CNF), anionic (a-CNF), and cationic (c-CNF). The native grade (u-CNF) was produced from hardwood pulp, which was converted to the sodium form prior to fibrillation. After ion exchange, the pulp was soaked at 1.7% consistency and dispersed using a high-shear Ystral X50/10 Dispermix mixer for 10 min at 2000 rpm. Next the pulp suspensions were prepared in a grinder (Supermasscolloidor MZKA10-15J, Masuko Sangyo Co., Japan) at 1500 rpm. The specific energy consumption in the prerefining was 2.0 kWh/kg dry pulp. The prerefining pulp suspension was supplied into a Microfluidics microfluidizer type M110-EH. The first pass was supplied through chambers having a diameter of 400 and 200 μm. The next nine passes were through 400 and 100 μm chambers. The gel was produced after ten passes, and the operating pressure was 1800 bar. The fiber slurry became a homogeneous and viscous gel after the mechanical treatment, with a final solid content of 1.5%.

The anionic grade (a-CNF) was produced from softwood kraft pulp using TEMPO-mediated oxidation. The chemical pretreatment was carried out according to the method applied by Saito et al.39 TEMPO (0.1 mmol/g) and NaBr (1 mmol/g) were used to catalyze the oxidation reaction with NaClO (5 mmol/g). The pH was kept at 10.5 by adding 1 M NaOH during the reaction. When the pH stopped decreasing, the reaction was stopped by adding the oxidized pulp suspension. Finally, the pH was adjusted to 7 by adding 1 M HCl. The oxidized pulp was washed with deionized water by filtration and stored in a fridge before filtration. The carboxyl content of the oxidized pulp was determined using conductometric titration according to the method described by the researchers. The oxidized pulp was soaked at 1.5% solids and dispersed using a high-shear Ystral X50/10 Dispermix mixer for 10 min at 2000 rpm. The pulp suspension was then fed into a Microfluidics microfluidizer type M110-EH at 1800 bar pressure. The suspension went twice through the chambers with diameters 400 and 100 μm. After the first pass, the gel was further diluted to 1% consistency and then fed into the microfluidizer the second time. The final product formed a viscous and transparent hydrogel with a final dry material content of 1.16% and a charge value of 1 mmol/g dry pulp.

The cationic grade (c-CNF) was made of hardwood kraft pulp. Cationization was conducted similarly to that of Bendoraitiene et al.,42 who reported the cationization of starch using EPTMAC (Raisacat, Chemigate) as a cationizing agent. The pulp was first concentrated in an oven to 63% dry matter content. The reaction mixture was prepared from 140 mL of Raisacat, 2 g of aqueous solution of NaOH (5%), and 2.3 mL of water. The ingredients were thoroughly mixed, and 50 mL of water was added to the mixture, which was warmed to 45 °C. The pulp (167 g) was added to the mixture, and it was stirred for 24 h at a high cellulose consistency with a CV Helicon Mix Flow (Design Integrated Technology USA, Inc.) reactor. After the reaction, the cationic pulp was washed with 500 mL of ethanol, 500 mL of tetrahydrofuran (THF), and 1000 mL of water. It was dispersed and fibrillated with the same method used with the anionic grade, but the fibrillation consistency was kept at 2%. The final product formed a highly viscous and transparent hydrogel with a final dry material content of 2.01%. The degree of substitution was analyzed according to Bendoraitiene et al. and was 0.35.

2.2.2. Preparation of CNF Surfaces. Samples of u-CNF, a-CNF, and c-CNF gels were prepared at 0.15 wt % (W/v) solutions in Milli-Q water and sonicated for 2 min at 20% amplitude using a SONICS Vibra cell VCX 750 ultrasonic processor (Sonics and Materials, Inc., U.S.A.). In order to remove large nanofibers, the sonicated samples were centrifuged at 10000 g for 60 min (Thermo Scientific SC SL) and the supernatant was used for the experiments.

Glass cover plates were cleaned by several immersions in 70% ethanol before coating. To achieve better adhesion between the CNFs and the glass, clean glass surfaces were coated with polyvinylamine (PVAm) by dispensing 300 μL of 0.5 mg/mL polymer solution on the surfaces, followed by a 15 min incubation at room temperature and washing with deionized water. PVAm-coated surfaces were not allowed to dry before adding 2 or 200 μL of 0.15 wt % sonicated and centrifuged u-CNF, a-CNF, or c-CNF solution. CNF surfaces were prepared by a droplet-drying process carried out by dropping 2 or 200 μL of the dispersions onto the glass cover plates as droplets of printing on the surface with the assistance of lines in a process similar to contact printing. Surfaces were dried in an oven (UNSS, Memcert GmbH + Co. KG, Schwabach, Germany) at 60 °C or at varying temperatures (37–60 °C) on top of an ITO heat plate (H401-Glass-Frame, OkoLab) compatible with an optical microscope and controlled with an H401-T controller (OkoLab). CNF-coated surfaces and cleaned control glass cover plate surfaces were exposed to ultraviolet light (UV) for 60 min before cell seeding. Triplicates of u-CNF, a-CNF, c-CNF, and the control surfaces were used in the characterization and cell experiments. In addition, triplicates of the corresponding surfaces were coated with fibronectin (FN) for comparison with the experiments. FN solution with a concentration of 10 μg/mL was applied to the triplicate u-CNF, a-CNF, c-CNF, and control surfaces for 60 min at room temperature. Thereafter, the FN solution was removed, and the surfaces were washed three times with PBS solution before cell culturing. The FN-coated surfaces were not allowed to dry before adding the cell suspension on the surfaces.

In addition to the droplet-casting method, c-CNF samples were dispensed on a surface in a process similar to contact printing. Preparation of the PVAm coating was performed as described above. PVAm-coated surfaces were not allowed to dry before dispensing a line of 2 μL of c-CNF solution using a pipet in contact with the coated glass cover plate surface placed on the top of the ITO heat plate; this was then allowed to dry at 60 °C in an exposure to UV for 60 min before cell seeding. The sample types used in the cell experiments are summarized in Table 1. The operation process of the evaporated CNF surfaces is illustrated in Figure 1.

### Table 1. Surfaces and Their Abbreviations Used in the Cell Culturing Experiments

<table>
<thead>
<tr>
<th>surface name</th>
<th>surface description</th>
</tr>
</thead>
<tbody>
<tr>
<td>u-CNF (FN)</td>
<td>unmodified native CNF, droplet casted</td>
</tr>
<tr>
<td>a-CNF (FN)</td>
<td>anionic, TEMPO-oxidized CNF, droplet casted</td>
</tr>
<tr>
<td>c-CNF (FN)</td>
<td>cationic CNF, droplet casted</td>
</tr>
<tr>
<td>or c-CNF</td>
<td>oriented c-CNF, contact dispensed</td>
</tr>
<tr>
<td>positive control (FN)</td>
<td>uncoated glass cover plate</td>
</tr>
<tr>
<td>negative control</td>
<td>uncoated glass cover plate, 5% DMSO in growth medium</td>
</tr>
</tbody>
</table>

**FN after the sample abbreviation denotes an FN coating on the corresponding surface. The samples were prepared in triplicate with or without the FN coating for each experiment, as described in the text.**

2.3. Characterization of u-CNF, a-CNF, and c-CNF Samples. The CNF grades used in this study have been previously characterized for turbidity, pH, consistency, conductivity, and shear viscosity as well as for dry fiber thickness using atomic force microscopy (AFM) as dry fibril width using field emission scanning electron microscopy (FE-SEM), and morphology of the stained samples using optical microscopy (OM).

2.4. Characterization of Droplet-Evaporated CNF Surfaces and Droplet Evaporation. 2.4.1. Atomic Force Microscopy (AFM). To observe the fiber dimensions and fiber arrangement on the droplet-evaporated CNF surfaces, AFM (XE-100, Park Systems, U.S.A.) scanning was performed in a tapping mode using a standard force microscopy probe (AFM, probe, U.S.A.) with a resolution of 256 × 256 pixels. Images were analyzed and postprocessed using AFM imaging processing.
software XEI (Park Systems, U.S.A.), MATLAB R2106a (The MathWorks, Inc., U.S.A.), and CytoSpectre.85 XEI was used to obtain the arithmetic average height parameter \((R_a)\) from several points \((n = 27)\) of the AFM images. Surface roughness was analyzed with MATLAB from selected AFM scans separately in horizontal (rows) and vertical (columns) directions. This resulted in 256 data groups \((n = 256)\) in each direction. A spacing parameter called high spot count (HSC), described elsewhere,86 was obtained from the horizontal and vertical roughness plots in order to distinguish the horizontal repetition over aligned and more random surfaces. In the HSC determination, we set the "selected level" parameter 86 as 10% of the height of the highest peak above the zero average line. The "average wavelength" \((\lambda_f)\) parameter86 was obtained to estimate the spacing and the length scale of the repetitive aligned structure. The fiber alignment was quantified using the spectral analysis of orientation method provided by the CytoSpectre software, described in section 2.6.

2.4.2. Helium Ion Microscopy (HIM). To observe the surface topography of the CNF surfaces used in the cell experiments, helium ion microscopy (HIM; Orion NanoFab, Zeiss, Germany) was conducted using a 45° or 60° angle between the sample surface and helium beam, a working distance (eucentric distance) of 8.9 or 19 mm, and 0.3 pA helium ion beam power. The electron beam produced by the flood gun was used to compensate for the charging of the sample surface. The image size was 2048 \(\times\) 2048 pixels, and a 1 \(\mu\)m dwell time and 128x averaging was used.

2.4.3. Optical Microscopy. The droplet evaporation of the c-CNF droplets (described in detail in section 2.4.5) as well as droplet-evaporated and contact-dispersed CNF surfaces was viewed with an optical microscope (Zeiss AxioObserver.Z1, Germany). The glass cover plates were placed on the ITO heat plate during drying of the CNF surfaces at controlled temperatures of 37, 42, 47, 52, and 57 °C. Post-processing of the pictures was done in ImageJ.

2.4.4. Contact Angle Measurement. Wetting of the u-CNF \((n = 8)\), a-CNF \((n = 12)\), and c-CNF \((n = 12)\) surfaces was analyzed by the sessile drop method, using an OCA-15 plus optical goniometer (Dataphysics Instruments GmbH, Germany). A 2 \(\mu\)L drop of deionized water was dispensed on the edge of the droplet-evaporated CNF surfaces (200 \(\mu\)L), and the resulting side profile photographs of the droplet was captured with the goniometer to determine the static contact angle.

2.4.5. Droplet Evaporation Dynamics. To observe the evaporation dynamics using image-based analysis, we conducted and recorded droplet evaporation. Evaporation was investigated from two view angles using an inverted microscope (Zeiss AxioObserver.Z1, Germany) and an OCA-15 plus goniometer (Dataphysics Instruments GmbH, Germany) in order to obtain detailed information on the evaporation. In order to understand the evaporation process of the c-CNF droplet, deionized water evaporation on PVAm coated and uncoated glass cover plate was investigated as controls. Water evaporation on glass provides a standard reference, while the water evaporation on the PVAm coating provides a control for the c-CNF evaporation on the PVAm coating. A heat plate (at 60 °C) was placed on a microscope stand. A glass cover plate was used as a substrate, as described in Preparation of CNF Surfaces and was treated accordingly. A total of 0.5 \(\mu\)L of 0.15 wt % c-CNF and deionized water droplets were applied on PVAm coated glass cover plates and on uncoated glass cover plates. Subsequently, the evaporation of the droplets was recorded under OM using frame intervals between 11 and 51 ms. Each experimental setup (c-CNF on PVAm and deionized water on glass and PVAm) was repeated at least three times. Image-based analysis was conducted on the recorded data to show the displacement of the contact line as a function of time (during evaporation).

The evaporation of the droplet was characterized by tracking and plotting the propagation of the right and left edges of the droplet toward the center of the droplet as a function of time. A dark droplet was clearly discernible from its light background, and a binary image of each frame was formed by appropriate thresholding. The edge was then located from each binary image with 0.5 pixel accuracy. The pixel size was 1.17 \(\mu\)m. The location of the center of the droplet was determined from the last frame in which the droplet was present before it vanished. A more detailed description is given in associated content H.

In the contact angle measurements using the goniometer, a 2 \(\mu\)L drop of 0.15 wt % c-CNF and deionized water was dispensed on the PVAm coated and uncoated glass cover plate substrates, and the resulting side profile photographs were recorded using a frame interval 1 s to enable a detailed analysis of the contact angle and the contact line behavior during evaporation. Each experimental setup (c-CNF on PVAm and deionized water on glass and PVAm) was repeated at least six times. The recorded data was used to determine the contact angle and the width of the droplet base (mm) as a function of time.

2.5. Cell Culture and Evaluation of Cell Response to CNF Surfaces. Mouse embryonic fibroblasts (MEFs) originally obtained from Wolfgang H. Ziegler (Hannover Medical School, Hannover, Germany) were cultured in DMEM high glucose medium supplemented with 10% (v/v) fetal bovine serum, 1% l-glutamine, 1% P/S (100 IU/mL penicillin, 100 \(\mu\)g/mL streptomycin) in a humidified atmosphere of 95% air and 5% CO\(_2\) at 37 °C. Cells were harvested using
a trypsin-EDTA treatment and counted using a Burker’s chamber. Cell experiments were performed in passages 15–20.

2.5.1. AlamarBlue Assay. The AlamarBlue Cell Viability Assay Reagent was used to quantify cellular metabolic activity and, thus, to evaluate the concentration of viable cells in a given sample. The metabolic activity (conversion of resazurin into resorufin) of cells adhered to the CNF surfaces was determined by the AlamarBlue assay by seeding the CNF surfaces with the cell suspension with a density of 1.2 × 10^5 cells/mL. As a negative control, cells were seeded at the same density in 5% DMSO. As a positive control, a glass cover plate surface was used. After 44 h of culturing, the CNF substrates were transferred to a new 24-well plate. Then, 50 μL of AlamarBlue solution and 450 μL of medium were added to the wells (tot. 500 μL) and incubated at 37 °C. 5% CO2 in a humidified atmosphere for 5 ± 1 h. The fluorescence intensity was read at a 560 nm excitation wavelength and 590 nm emission wavelength by using a spectrophotometer (Envision UV/vis, PerkinElmer). The results are expressed in arbitrary units and reported as mean value ± standard deviation of the mean for n = 3. In addition, nanocellulose surfaces without cells were exposed to the AlamarBlue reagent to confirm that there was no interaction between the AlamarBlue reagent and nanocellulose surfaces. Hua et al.13 reported no interactions between AlamarBlue and their nanocellulose films, and no interactions were detected in this study.

2.5.2. Characterization of Cellular Growth Using Optical Microscopy. In addition to cell viability, tested with the AlamarBlue reagent, cell adhesion, spreading, and proliferation, as well as cell morphology on the CNF surfaces, were viewed using a live cell imaging system providing stable incubation at 37 °C and 5% CO2 (Cell-IQ CM Technologies, Finland). Approximately 1.2 × 10^5 cells/mL were plated on triplicate u-CNF, a-CNF, c-CNF, and control surfaces and incubated in Cell-IQ for 48 ± 2 h, while cells were observed and tile images were recorded. Tile matrices (3 × 3 or 4 × 4) covered either the entire area of evaporated droplets or a subset of this area, the contact-dispensed lines, or the control surfaces, and each tile image was recorded from a predetermined position approximately every 30–90 min. After the experiments, the cell numbers, proliferation, and cell morphology were evaluated from the images obtained using the optical microscope in the Cell IQ imaging system. The number of living cells was manually counted from the optical microscope images to estimate cellular growth and proliferation. The cell number was calculated from nanocellulose and reference surfaces 2 h after plating and after a 48 h cultivation period. The ratio of the cell numbers after 2 and 48 h culturing periods was reduced from one, and the obtained value describing the cell proliferation was compared for different surfaces and with the cell viability results. The closer the value is to one, the better the cell proliferation. The cell numbers were calculated from at least 12 images per time point.

2.6. Quantification of Cell and CNF Alignment. A spectral orientation analysis tool (CytoSpectre®) was used to characterize the degree of alignment of the cells on the CNF and control surfaces. CytoSpectre is a software tool for the analysis of orientation and wavelength distributions from micrographs. The software utilizes the Fourier transform to estimate the power spectrum of an image and, based on the spectrum, computes parameter values describing, among others, the mean orientation and isotropy. Here, circular variance (CV) values are used to determine the degree of cell orientation from micrographs obtained from live cell imaging experiments and the degree of fiber orientation from AFM amplitude images of CNF surfaces. A circular variance is a measure of the isotropy of the orientation distribution, the value zero corresponding to perfect alignment of all oriented structures along a single line and the value one corresponding to a perfect lack of a dominant orientation. In addition to CV values, orientation plots are obtained from the software in order to point out the orientation direction in the example images.

3. RESULTS AND DISCUSSION

An evaporation-induced droplet-casting method was investigated in order to produce self-aligned CNF surfaces. First, we report the previously determined properties of u-CNF, a-CNF, and c-CNF grades. Next, we show the degree of alignment of the corresponding droplet-evaporated CNF surfaces and explain the alignment mechanism based on the investigations of the droplet evaporation. Finally, we show cell viability, growth, morphology, and degree of alignment on the droplet-evaporated CNF surfaces. We also report the possibility of controlling the orientation of the c-CNFs and thus the cells by altering the shape of the evaporating boundary line of the c-CNF solution.

3.1. Characterization of CNF Grades. Previously determined turbidity, pH, consistency, conductivity, and shear viscosity of the CNFs used in this paper are summarized in Table 2 and discussed in this section.

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<th>Table 2. Characterization Card of CNF Grades</th>
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$^*$HW = hardwood, SW = softwood. $^b$NTU scale 0–1000 NTU. $^c$Anton Paar MCR301, ST22–4 V vane spindle, consistency 0.5%, T = 23 °C.

CNF grades were previously characterized using OM and scanning electron microscopy (SEM). Results are summarized here to provide an overview of the samples, and later to interpret the results obtained in this study. An overview of the u-CNF, a-CNF, and c-CNF samples, macrostructures, and homogeneities were obtained using an optical microscope. A stained u-CNF sample (Figure 2a) appears with some unrefined fibers, while unrefined fibers are not observed in the a-CNF sample (Figure 2b), indicating a more homogeneous sample. The c-CNF sample (Figure 2c) possessed unrefined fibers, larger fiber fragments, and fibrous particles with small dimensions providing a more coarse and heterogeneous surface. Turbidity values (Table 2) of the CNF grades confirm the observations made with the optical microscope, indicating that the fibril widths are largest in u-CNF samples and smallest in a-CNF samples. The previously performed AFM image analysis revealed increasing dry fibril thickness from c-CNF to a-CNF to u-CNF samples. For u-CNF the mean fibril thickness values have been between 20 and 30 nm and for the c-CNF and a-CNF between 10 and 15 nm. These results are in line with our SEM observations (Figure 3). In general, the higher the fiber charge, the smaller the fibril diameter of a-CNF.

Previous SEM images provide an overview of the structural and dimensional appearance of u-CNF, a-CNF, and c-CNF samples. The u-CNF sample (Figure 3a) formed porous and bulkier fibrillar networks and possessed a larger fibril width than the a-CNF (Figure 3b) and c-CNF (Figure 3c) samples, which both formed a smoother layer of fibrils, confirming the OM studies. The finer fibril structure and water removal properties of the charged samples could result in a smoother film formation.

3.2. Characterization of Droplet-Evaporated CNF Surfaces. 3.2.1. Spectral Orientation Analysis from AFM Images. AFM scanning was performed on u-CNF, a-CNF, and c-CNF surfaces (Figure 4) to obtain information on the fiber arrangement and surface topography of the droplet-evaporated
surfaces. Besides visual inspection, fiber alignment was evaluated from the AFM images using two different techniques: spectral analysis of orientation and quantitative analyses of surface roughness plots.

Aligned fibers were detected on u-CNF (Figure 4a) and c-CNF (Figure 4e) surfaces, while the observed orientation was lower on the a-CNF surface (Figure 4c), an observation that was confirmed with the orientation plots of the corresponding surfaces and the average CV values, provided in Figure 4g. Orientation plots confirm the orientation direction, while CV values describe the degree of orientation. As seen from previously obtained shear viscosity values of the CNF grades (Table 2), u-CNF and c-CNF samples are less viscous, which might have an influence on fiber alignment during droplet evaporation.

The surfaces were scanned from several local regions outside the center area and in the center region of the evaporated c-CNF droplets, referred to as the peripheral and center areas, respectively, later in the text. Examples of the scanned areas and the corresponding AFM amplitude images are presented in Figure 5. A higher degree of orientation (average CV 0.27) was detected when scanning was performed on the peripheral area of the evaporated droplet on the c-CNF surface, than on the center area (average CV 0.64). A higher degree of orientation in the peripheral area may be a result of the alignment of the fibers parallel to the contact line during droplet evaporation, as shown in the sketch of an evaporated droplet presented in Figure 5; this will be discussed later.

3.2.2. Quantitative Analysis of Surface Roughness Plots. The arithmetic average height parameter ($R_a$) value was used to describe the overall roughness of the droplet-evaporated surfaces. The $R_a$ values presented in Table 3 indicate relatively smooth surfaces, especially in the peripheral area of the evaporated droplets. In addition to differences in the fiber arrangement between the peripheral and center areas of c-CNF surfaces (Figure 5), $R_a$ values obtained in the center area of the c-CNF surfaces (Table 3) indicate variation between the peripheral and center areas also in respect to the surface roughness.

In addition to the spectral analysis of the orientation, fiber alignment was evaluated using a quantitative analysis (Figure 6) of surface roughness plots obtained from AFM images of aligned c-CNF (Figure 6a) and more random a-CNF (Figure 6c) surfaces. An AFM image of the a-CNF (Figure 6c) surface with a lower degree of orientation represents a control surface. Surface roughness plots obtained from AFM images of the aligned c-CNF surface (Figure 6b) were different in the directions parallel to the assumed fiber orientation (horizontal) and perpendicular to the fiber orientation (vertical). However, surface roughness (measured with $R_a$) was similar in both directions, 5.67 and 5.30 nm, respectively (Table 3); the peaks in the roughness plot appeared more frequently perpendicular to the assumed alignment than parallel to the assumed alignment (Figure 6b).

The observed differences in the roughness plots parallel and perpendicular to the assumed alignment could be the result of aligned and tightly packed fibers. Such a difference was not detected when control roughness plots (Figure 6d) were analyzed. The spacing parameter HSC was obtained from the roughness plots in order to distinguish a possible periodicity over the aligned c-CNF surface versus the control a-CNF surface. The HSC obtained from roughness plots ($n = 256$) in both horizontal and vertical directions (Figure 6f) confirmed the periodicity over the aligned surfaces. The average HSC (Figure 6g) is 1.93X higher in the roughness plots of the c-CNF surface in the direction perpendicular to the assumed fiber direction than parallel to the assumed fiber alignment, which could be a result of evenly aligned and tightly packed fibers. The corresponding ratio of the average HSC for the control is 1.28, indicating a smaller difference in the roughness plots. Similar results were obtained with a quantitative analysis conducted using the average wavelength parameter ($\lambda_a$; Figure 6g,h), which is a measure of
the spacing between local peaks and valleys, taking into consideration their relative amplitudes and individual spatial frequencies.\(^\text{39}\) From the curves (Figure 6g) we can also see smaller variations through the image \((n = 256)\) perpendicular to alignment, indicating regular spacing between the adjacent peaks, and thus regularly arranged fibers. Average \(\lambda_a\) (Figure 6h) is 2.02× higher in the roughness plots of the c-CNF surface parallel to alignment than in that perpendicular to alignment. This is because fibers are more tightly packed perpendicular to alignment and therefore the spacings between adjacent peaks in the roughness plot are closer to each other. The corresponding ratio of average \(\lambda_a\) for the control is 1.27, indicating a smaller variation in the distance of the peaks in the control roughness plots. The parameter \(\lambda_a\) thus allows us to obtain a rough estimate of the size of the periodic structure in the aligned sample, which is approximately 65 nm.

3.2.3. HIM Imaging. HIM imaging provided an overview of the surface topography and the cross-section of the droplet-evaporated u-CNF, a-CNF, and c-CNF surfaces (Figure 7). Smooth u-CNF (Figure 7a) and c-CNF (Figure 7g) surfaces detected from the surface roughness results (Table 3) were confirmed using HIM. Detection of the alignment on c-CNF surfaces (Figure 7h) was challenging due to the smoothness of the surfaces, with low surface roughness values and tightly packed fibers, which result in poor contrast in HIM images of the aligned surfaces. A crystalline surface is observed in the center area of the a-CNF sample (Figure 7d–f). The cross sections of scratched u-CNF (Figure 7b,c) and c-CNF (Figure 7i) surfaces indicate that these surfaces consist of several CNF layers. When scratched, u-CNF and c-CNF coatings stratify without degrading and form ordered folds, visible in the HIM images (Figure 7b,c,i).

3.3. Mechanism of Droplet Evaporation and Subsequent Alignment of the Fibers along the Boundary Line. In order to explain the phenomenon behind the observed fiber alignment, droplet evaporation of a c-CNF solution was imaged under an optical microscope. The aim was to observe whether evaporation occurs with a pinned or unpinned boundary line,
knowledge that is needed to explain the governing alignment phenomenon during droplet evaporation. A pinned boundary line would cause the formation of a “coffee ring” deposit, which is a result of the outward capillary flow during water evaporation. Alternating pinning and de-pinning of the line, also referred to as “stick−slip” motion, would result in the formation of concentric rings after complete evaporation of the solution.

The evaporation of the droplets on the heat plate (at 60 °C) are shown in videos A−E (associated content), reconstructed from the side profile images (Videos A, B, and C) recorded with a goniometer, and from inverted microscopy images (Videos D and E). In addition, snapshots from the videos are represented in Figures 8 and 9, respectively. Analysis results from the recorded images are presented in Figures 10 and 11. While Figure 10 shows the contact angles and the diameters of the contact bases of the droplets, Figure 11 presents the displacement of the droplet contact line as a function of time.

The constant contact line (CCL) mode (described in ref 87) observed in the beginning of evaporation is similar in all studied droplets, as detected from the contact base diameter values recorded from the droplet side profiles (Videos A−C in associated content, and Figures 8 and 10). The constant contact line is apparent also in the displacement plots of the contact lines in Figure 11. A decrease in the contact angle is detected simultaneously with the constant position of the contact line (Figure 10). Thus, in the beginning the contact angle is changing while the location of the contact line remains constant. However, differences in the contact line behavior arise as the contact line begins to move. The evaporation of c-CNF droplets is remarkably different from that of the water droplets after the contact line begins to move. Relatively steady displacement of the c-CNF contact line is observed through the evaporation (Video D in Associated content). After the CCL mode, we observe no constant contact angle (CCA) mode in the
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Figure 6. AFM amplitude images of (a) c-CNF (aligned) and (c) a-CNF (oriented) surfaces. Surface roughness plots obtained from the center area (0.8 μm) of the corresponding AFM scans, aligned (b) and control (d), in the horizontal and vertical directions, respectively. Peaks of the roughness curves are determined according to the rules described in Materials and Methods. The HSC selected level parameter is added to the roughness plots in which 0 corresponds to the average value. (e) HSCs obtained from all vertical (n = 256) and horizontal (n = 256) roughness plots. (f) Average HSC (n = 256) and standard deviations. (g) Average wavelength (λ) parameters obtained from all vertical (n = 256) and horizontal (n = 256) roughness plots. (h) Average of λ (n = 256) and standard deviations.

Figure 7. HIM images of u-CNF (a−c), a-CNF (d−f), and c-CNF (g−i) surfaces. A pleated c-CNF structure on the edge (top right corner) of the evaporated droplet (g), and a scratched c-CNF surface with a pleated structure (i). The CV value in (h) is 0.40.

Figure 8. Snapshots captured during evaporation using goniometer. (a) c-CNF on PVAm (video A), (b) deionized water on PVAm (video B), and (c) deionized water on glass (video C).

evaporating c-CNF droplet (Figure 10a). Instead, the contact angle slightly increases after it has reached its minimum value, indicating a mixed mode. Deionized water droplets, however, undergo the CCL-CCA transition very clearly, which is observable from Figure 10b,c. The contact line is not moving (i.e., the height of the droplet decreases), and the contact angle...
decreases in the CCL mode. In the CCA mode, the contact line is moving and thus the contact base diameter decreases, and the contact angle remains relatively unaltered (Figure 10b,c). As can be seen from Figure 10, the CCL → CCA transition is more distinct for the evaporating deionized water droplet on uncoated glass (Figure 10c) compared to that on PVAm-coated glass (Figure 10b).

The evaporation dynamics is significantly different between the c-CNF and deionized water droplets on PVAm coated glass. Videos D and E (in associated content) and the corresponding image-based analyses (Figure 11) depict the difference in the contact line movement of the c-CNF and deionized water droplets, as the c-CNF droplet undergoes controlled shrinking toward the center, while the contact line of the deionized water droplets experience severe pinning in random places of the
contact line. This can be seen from the displacement curves (Figure 11b,c), as the right-hand edge remains closer to the final center point than the left-hand edge. This is shown very clearly in video E (associated content). The right-hand edge of the droplet in these cases (Figure 11b,c) is pinned for long time, and the droplet shrinks toward this edge of the image, which is also the reason for the defined center pixel to appear outside the original center of the applied droplet. Controlled contact line movement is likely to be due to higher viscosity and higher surface tension of the c-CNF droplets compared to that of deionized water. However, the effect of surface tension, droplet concentration and surface roughness requires further investigations in order to explain the evaporation mechanisms in more detail.

Other studies report the formation of a “coffee ring” during the evaporation of CNC droplets but a lower degree of depinning events during the evaporation of CNF droplets. Our investigations on CNF droplet evaporation are in accordance with these studies, when CNF depinning events are considered. Real-time recordings and image-based analysis show a moving boundary line. However, local pinning of the contact line caused by larger fibers can be observed from video D (associated content). This pinning is not, however, relevant, as the droplet continues its controlled shrinking after passing the larger fiber. In order to detect even the slight pinning events of the moving water-CNF contact line, all recorded frames captured with OM (at 11 ms interval) were analyzed and the distance versus time curve was zoomed into the pixel level. No pinning events were detected in the 11 ms frame analysis.

Figure 12. Optical microscope images of droplet-evaporated c-CNF surfaces at 37 °C (a) and at 57 °C on an ITO heat plate (b). Center area of the evaporated c-CNF surface (c). Ordered c-CNF surface incubated in DMEM (d–f). Contact-dispensed surfaces (g–i) evaporated at >80 °C.
cell experiments was conducted at 60 °C, which still provided aligned straight lines.

3.4. Alignment Mechanism. In this study, the alignment mechanism is similar to that of Maskhour et al.,77 who studied CNCs, also called cellulose nanowhiskers. That study explains that the self-assembly of CNCs is related to the formation of a surface tension torque close to the dry-line boundary layer during evaporation of a CNC suspension, where the surface tension torque is suggested to align CNCs tangential to the dry-line boundary layer. Surface tension induces a rotating moment that might affect the orientation of nanoparticles in suspension in the proximity of the dry-line boundary and align nanoparticles parallel to the dry-line boundary. The surface tension gradient and surface evaporation are the two main driving forces close to the dry-line boundary; they are thought to act on the anisotropic CNCs, resulting in surface tension torques proportional to the length of the CNCs and the contact angle between the CNCs and the surface of the glass substrate, rotating the CNCs and aligning them parallel to the dry-line boundary layer.77 Surface tension torque that affects the particle and aligns it is given by the following equation described in ref 77

$$\tau = F_s \sin(\pi/2 - \beta)$$

where $l$ is the length of the particle meaning that longer particles are subject to the higher surface tension torque. $F_s$ is the magnitude of the surface tension force of the substrate. $\beta$ is the angle between the long axis of the affected particle and the dry-line boundary layer, and its magnitude is variable between zero to $\pi/2$.77

We hypothesize that in the case of CNFs, the alignment process could be similar to that proposed for CNCs, with some variation due to the CNFs’ longer length, which could limit the rotation of the nanoparticle. During the observed CCL mode of evaporation, the droplet volume decreases, and the CNFs will be subject to the Brownian motion of water molecules, which increases the local concentration of CNFs at the droplet edge. This was also described in the case of clay nanotubes,76 which have particle lengths closer to those of the CNFs than the smaller CNCs have. When CNFs are close to the triple line, the surface tension torque acts on them. It is energetically favorable that the CNFs will be located at the triple line as the surface free energy of the suspending liquid reduces upon increase in CNF concentration.77 The increased local concentration of CNFs at the droplet periphery may align the CNFs parallel to the droplet edge. The other end of the CNF chain could be pinned by the contact line in CCL mode in the beginning of the evaporation, which results in geometrical constraints and a change in the axial flow direction of the fiber parallel to the edge. This is probably due to the frictional force between the substrate and the fibers in suspension, coupled with the accelerated evaporation at the boundary line. Thus, surface tension-induced and flow-induced torque causes the axial flow direction of the CNFs to orientate parallel to the edge in the periphery of the droplet, aligning the first CNFs. The subsequent alignment is likely caused by the capillary forces induced by the water during evaporation, as the fibers tend to adhere to and align with adjacent fibers.60 Cationically charged groups in the backbone of the cellulose nanofibers make the fibers more hydrophilic, which strengthens the capillary forces that pull adjacent fibrils closer to each other during drying. Hua et al.56 suggested that stronger capillary forces were enhancing fibril alignment when anionically charged CNFs were vacuum filtered, and that this could be related to entropic effects and reeding average distances between adjacent fibrils as well as an increasing interfibrillar contact area.

In addition to particle concentration, length, surface charge, pH, salt concentrations, and temperature,76 substrate properties, such as roughness, wetting, and surface tension, most likely affect evaporation and alignment. Therefore, in order to understand the mechanism controlling the alignment of CNFs during droplet evaporation, a wider investigation is yet required.

The self-orienting capacity of CNFs is not a well-described phenomenon. Alignment during evaporation-induced self-

Figure 13. Optical microscope images of MEFs cultivated on the positive control (a), u-CNF (b), negative control (c), a-CNF (d), c-CNF (e), and oriented c-CNF (f) surfaces after 48 h of cultivation. Surfaces of u-CNF (b), a-CNF (d), and c-CNF (e) were droplet evaporated, while the oriented c-CNF surface (f) was contact dispensed.
assembly has been observed with CNCs\textsuperscript{77,88,89} but not with CNFs\textsuperscript{88,89}. When analyzing the results of other studies, differences in nanocellulose origin, extraction techniques, modifications, and experimental setups have to be considered. A thorough understanding of the drying process of the nanoparticle solution, and a controlled manipulation of the shape of the evaporating dry-line boundary, allow the controlled assembly of various particles into intriguing, well-structured, and well-aligned patterns\textsuperscript{60}. Mashkour et al.\textsuperscript{77} suggested that surface tension torque negatively affects attempts to align CNCs under magnetic and electric fields and that a considerable amount of energy would be required to align CNCs under these fields, to overcome the surface tension torque when a liquid suspension evaporates to form the final film; also, the final alignment would often be unsatisfactory. Similar challenges might arise during active CNF alignment due to simultaneous forces acting on the self-aligning of CNFs.

**3.5. Cell Response to CNF Surfaces.**

**3.5.1. Cell Proliferation on CNF Surfaces.** Cell growth was examined on droplet-evaporated u-CNF, a-CNF, and c-CNF surfaces and the corresponding FN-coated u-CNF (FN), a-CNF (FN), and c-CNF (FN) surfaces, as well as on the contact-dispersed c-CNF surface. Cell numbers were manually determined from live cell images taken 2 h after plating and after 48 h (Figure 13) of culturing. The results (Cell IQ proliferation data) in Figure 14 show that MEFs cultured on the u-CNF, u-CNF (FN), and negative control surfaces (5% DMSO) had poor viability and did not proliferate during the 48 h cultivation period. The round cell morphology observed on the u-CNF (Figure 13b) and negative control (Figure 13c) surfaces indicates poor attachment on both surfaces and therefore poor viability. However, the cell growth was significantly improved on the positive control, a-CNF, and c-CNF surfaces, as well as on the corresponding FN-coated surfaces (Figure 14). MEFs on the oriented c-CNF surface showed the highest proportional increase in cell number.
even more increased spreading (Figure 13e,f). The FN-coated surfaces showed increased spreading in some areas. Cell morphology on the c-CNF surfaces showed the positive control surface, they showed increased spreading in all FN-coated surfaces compared to the corresponding surfaces without the FN coating. MEFs cultured on the positive control surface presented a round but not sufficiently adhered shape (Figure 13a). However, while cells cultured on a-CNF surfaces (Figure 13d) are similar in morphology to those on the positive control surface, they showed increased spreading in some areas. Cell morphology on the c-CNF surfaces showed even more increased spreading (Figure 13e,f). The FN-coated surfaces followed the same trend as the corresponding uncoated surfaces.

3.5.2. Cell Viability on CNF Surfaces. Viability of MEFs on different CNF surfaces was investigated using the AlamarBlue assay. The AlamarBlue results, shown in Figure 15, showed a trend similar to the one found with the proliferation data obtained with Cell IQ (Figure 14). Poor viability was observed on the u-CNF surface; viability significantly increased on the u-CNF (FN) surface, but still remained lower than the values for the positive control. The number of viable adherent cells found on the surfaces of the c-CNf and a-CNf samples, as well as on the corresponding FN-coated surfaces, were comparable with the values found for the positive control, thus indicating an improved biocompatibility of the surfaces covered with charged CNFs (a-CNf and c-CNf) compared to those covered with u-CNf.

No additional reactions between AlamarBlue and CNF surfaces were observed when AlamarBlue was incubated with the CNF surfaces alone. Cell viability as well as proliferation was slightly enhanced in all FN-coated surfaces compared to the corresponding surfaces without the FN coating. The charged a-CNF and c-CNF surface was found to be comparable to the values found for the positive FN-coated control, followed by the cells seeded on the FN-coated a-CNf, FN-coated c-CNf, positive control, a-CNf, and c-CNf surfaces. According to the slight increase in cell numbers, proliferation was slightly enhanced in all FN-coated surfaces compared to the corresponding surfaces without the FN coating. MEFs cultured on the positive control surface presented a round but not sufficiently adhered shape (Figure 13a). However, while cells cultured on a-CNf surfaces (Figure 13d) are similar in morphology to those on the positive control surface, they showed increased spreading in some areas. Cell morphology on the c-CNf surfaces showed even more increased spreading (Figure 13e,f). The FN-coated surfaces followed the same trend as the corresponding uncoated surfaces.

AlamarBlue assay indicates some metabolic activity in the u-CNf, u-CNf (FN), and negative control samples, indicating that there are live cells in these samples. However, a microscopic observation in Cell IQ revealed a round, poorly attached cell morphology on the u-CNf and negative control surfaces (Figure 13b and c, respectively) with no proliferation of cells; the cell number did not increase during the 48 h cultivation period (Figure 14). In summary, the AlamarBlue results showed some metabolic activity, indicating that the cells were still alive, but microscopic observations showed that they were poorly attached. These results demonstrate, as recommended elsewhere, that with AlamarBlue assays it is important to simultaneously monitor cell growth with an optical microscope.

The cell numbers on the a-CNf, c-CNf, positive control, and corresponding FN-coated surfaces corresponded to the results obtained from viability tests, showing similar increases in cell number and metabolic activity when uncoated and FN-coated surfaces were compared, and when different CNF surfaces (either FN-coated or uncoated) were compared with each other and with positive control surfaces. Results indicate a slight but observable enhancement of cell metabolic activity and proliferation caused by the FN coating. The similar results of the proportional increase in cell number and the amount of metabolic activity (measured with AlamarBlue) indicate the reliability of results obtained with these two complementary methods in the cases of a-CNf, c-CNf, positive control, and corresponding FN-coated surfaces.

A slight reduction in cellular proliferation was detected in most of the CNF surfaces compared to the positive control surfaces, as judged by proliferation analyses and AlamarBlue assays. Our results are comparable to those of other studies, in which different CNF surfaces were found to be suitable substrates for cell cultivation but with a slight reduction in cell proliferation. However, both Čolić et al. and Moreira et al. reported that the cells cultivated with CNFs preserved their morphologies. Here, we obtained significant cell elongation on CNF surfaces, discussed more in section 3.5.4, which is the most significant difference compared to other studies.

The charged a-CNf and c-CNf surface was found to be suitable for cell cultivation (Figures 13–15). Hua et al. studied surface modification effects, that is, changes in CNF surface
topography, chemistry, and charge during biological responses of human dermal fibroblasts and demonstrated that negatively charged CNF substrates promoted fibroblast adhesion and spreading and presented a more cytompatible profile than positively charged and unmodified CNF surfaces. However, different studies should be compared with caution due to differences in nanocellulose origin, CNF preparation, substrate preparation, and the studied cell lines, among other factors.

3.5.3. Influence of Material Properties on Cell Growth. To explain the differences observed in cell viability, cell growth, and cell morphology on the various CNF surfaces studied is a complex task. In general, there are several aspects that affect cell adhesion to artificial materials, such as wettability, roughness, surface charge, and chemical functionalities. The dynamic contact angles of the u-CNF, a-CNF, and c-CNF surfaces used for the cell experiments were 32.56, 29.64, and 67.70, respectively, which alone does not explain the differences in cell growth. The remarkably higher contact angle value on the c-CNF surface may have an effect on the adhesion and spreading of the cells, but this was not studied here at a molecular level.

The surface roughness differences between the u-CNF, a-CNF, and c-CNF surfaces were relatively small (Table 3). The surface changes of the u-CNF, a-CNF, and c-CNF surfaces most probably differ from each other, as the reported zeta potential values for the corresponding grades are approximately −25, −69.5, and +41 mV, respectively. In addition, there are differences in chemical functionalities due to the anionic and cationic groups substituted to the anhydroglucose units in the a-CNF and c-CNF samples, respectively. Fiber dimensions, morphology, and arrangement have an effect on cell adhesion. An aligned CNF structure, it has been suggested, improves cell adhesion. Further studies are required at a molecular level to explain cell adhesion on the studied CNF surfaces in detail.

3.5.4. Cell Orientation on Aligned c-CNF Surfaces. The morphology and orientation of the cells on different CNF surfaces were investigated using an optical microscope (Figure 13) and spectral orientation analysis (Figure 16). Cells aligned in a circular pattern on the droplet-evaporated c-CNF surfaces (Figures 13e and 17e and video F, provided in the associated content). Cell alignment is clearly detectable in the peripheral areas of the central c-CNF surfaces, while cells in the central area remain in a random orientation (Figure 17d–f). This accords with our suggestion that CNFs align parallel to the evaporation line and remain random in the center of the evaporated droplet. As an attempt to control the orientation direction, a contact-dispensing c-CNF solution was used to make a straight c-CNF line, resulting in aligned c-CNFs and thus unidirectionally aligned cells peripheral to the c-CNF evaporation line axis (Figures 13f and 16c). The proliferation and orientation of the cells parallel to the boundary line are shown in videos F and G (associated content), reconstructed from the microscope images during the 48 h cultivation period in Cell IQ. The possibility of controlling the orientation direction of the cells is a key finding of this study.

We analyzed the cell orientation more quantitatively using spectral analysis and obtained results comparable to those observed in the microscope images (Figures 13 and 16). On the a-CNF surface and on the positive control surface, no significant degree of orientation was present, as seen in the microscope images (Figure 13a and d, respectively), and the average circular variance values were 0.89 (n = 19) and 0.93 (n = 9), respectively (Figure 16a). On the c-CNF surfaces, the orientation is clearly visible in the microscopic images (Figure 13e,f), and the average circular variance value is 0.39 (n = 33; Figure 16a). In addition to the average circular variance values, Figure 16 presents example images with randomly oriented cells on the control surface and

Figure 17. AFM amplitude images (1 μm × 1 μm) from the peripheral area (a) with a circular variance of 0.38 and at the center area of the evaporated droplet (c) with a circular variance of 0.85. Cells cultivated on top of the peripheral (d) and center (f) areas. Sketched CNF alignment direction parallel to the dry boundary line as the droplet evaporates (b) and the corresponding OM image of cell orientation along the aligned CNFs (e).
oriented cells on the c-CNF surfaces (Figure 16b and c, respectively), and the corresponding schematic orientation plots provided by the Cytospectre tool (Figure 16d and e, respectively).

The schematic in Figure 17b summarizes the assumed periodic topography of the droplet-evaporated c-CNF surface and the corresponding orientation of the cells according to the evaporated droplet shape (Figure 17e). The c-CNF surface and therefore the cells align according to the dried boundary line of the evaporated droplet in the peripheral areas (Figure 17a and d, respectively), while CNFs and thus the cells in the central part of the dried area remain randomly distributed (Figure 17c and f, respectively).

4. CONCLUSIONS

We obtained aligned CNF surfaces by an easy droplet-evaporation method. We propose that the alignment is based on the effects of surface tension torques and capillary forces during evaporation. We showed that the evaporation of c-CNF droplets significantly differ from the evaporation of deionized water droplets, after the CCL mode in the beginning of evaporation. Further studies of the alignment and evaporation process will be important in order to understand the phenomenon and achieve more controlled oriented structures.

Although a 3D structure is often required to guide cell growth, even a single oriented layer can be used to initiate the alignment of specific cell types. Alignment could be guided through several layers by a single aligned basement surface. Aligned CNF surfaces have potential applications in tissue engineering; for example, they have applications in the formation of an organized fibroblast layer as feeder layers in stem cell applications; in the growth guidance of a specific cell type, and 3D scaffolds. They also have applications in coatings of implant materials; in wound healing applications; and in studies of cell signaling mechanisms. Aligned CNF substrates are extremely interesting substrates for tissue engineering since CNFs can be enzymatically degraded. In addition to the biomedical applications, the orientation of CNFs is desirable in composites to improve the stiffness and strength of the material. The described alignment method is not limited to a specific application.

In this paper, we also proved that cell alignment occurs on the aligned c-CNF surfaces and showed that the evaporation-induced droplet-casting method is an appealing and effortless method to create cell-aligning substrates. We also studied the effect of different CNF surfaces on cell responses. The cell responses were studied by proliferation analyses obtained from Cell IQ live imaging experiments, and by the resazurin reduction to resorufin indicating cell viability (AlamarBlue assays), morphological changes of cells in the cultures, and cell proliferation.

The cells either attached and aligned, attached and preserved a similar morphology to that in the positive control surface, or did not attach on the CNF surfaces. Based on these results, we can conclude that a-CNF and c-CNF surfaces can be used as cell culturing substrates, the latter promoting cell alignment. u-CNF is unsuitable for cell culturing, although adding functional or biocompatible groups via the OH groups of the cellulose backbone might improve its cytocompatibility.

We also demonstrated that cells can be cultivated directly on charged CNF surfaces without the need of a protein biocompatibility layer, although an FN coating slightly increased cell viability and proliferation, compared to the CNF surface without the protein coating.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.7b00963.

Video, c-CNF evaporation on PVAm, side view (AVI). Video, deionized water evaporation on PVAm, side view (AVI).

Video, deionized water evaporation on glass, side view (AVI). Video, c-CNF evaporation on PVAm, bottom view (AVI). Video, deionized water evaporation on glass, bottom view (AVI).

Video, fibroblast growth (48 h) on droplet-evaporated (2 μL) c-CNF surface (AVI).

Video, fibroblast growth (48 h) on evaporated contact-dispersed c-CNF surface (AVI).

Description of image-based analysis (PDF).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. A.S. contributed in the planning, performance and reporting of the experimental work, excluding HIM imaging. A.-J.M. contributed in quantitative analysis. M.M. contributed in image-based analysis. P.L. contributed in CNF preparation and providing the previous CNF characterization results. P.K. contributed in the study conception, coordination of the work, and manuscript structuring and editing.

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Notes

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respectively), and the corresponding schematic orientation plots of specific layers by a single aligned basement surface. Aligned CNF surfaces of specific water droplets, after the CCL mode in the beginning of during evaporation. We showed that the evaporation of c-CNF of different CNF surfaces on cell responses. The cell significantly differed in the positive control surface, or did responses were studied by proliferation analyses obtained from different cell type, and 3D scaffolds. They also have similar morphology to that in the positive control surface, or did not respond to the different surface properties. We also demonstrated that cells can be cultivated directly on edges for providing the cells, biomolecules, and advice in cell and similar morphology to that in the positive control surface, or did respond to the different surface properties. We also demonstrated that cells can be cultivated directly on edges for providing the cells, biomolecules, and advice in cell and similar morphology to that in the positive control surface, or did not respond to the different surface properties. We also demonstrated that cells can be cultivated directly on edges for providing the cells, biomolecules, and advice in cell and similar morphology to that in the positive control surface, or did not respond to the different surface properties.
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Cellulose Mesh with Charged Nanocellulose Coatings as a Promising Carrier of Skin and Stem Cells for Regenerative Applications


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Cellulose Mesh with Charged Nanocellulose Coatings as a Promising Carrier of Skin and Stem Cells for Regenerative Applications

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ABSTRACT: Engineering artificial skin constructs is an ongoing challenge. An ideal material for hosting skin cells is still to be discovered. A promising candidate is low-cost cellulose, which is commonly fabricated in the form of a mesh and is applied as a wound dressing. Unfortunately, the structure and the topography of current cellulose meshes are not optimal for cell growth. To enhance the surface structure and the physicochemical properties of a commercially available mesh, we coated the mesh with wood-derived cellulose nanofibrils (CNFs). Three different types of mesh coatings are proposed in this study as a skin cell carrier: positively charged cationic cellulose nanofibrils (cCNFs), negatively charged anionic cellulose nanofibrils (aCNFs), and a combination of these two materials (c+aCNFs). These cell carriers were seeded with normal human dermal fibroblasts (NHDFs) or with human adipose-derived stem cells (ADSCs) to investigate cell adhesion, spreading, morphology, and proliferation. The negatively charged aCNF coating significantly improved the proliferation of both cell types. The positively charged cCNF coating significantly enhanced the adhesion of ADSCs only. The number of NHDFs was similar on the cCNF coatings and on the noncoated pristine cellulose mesh. However, the three-dimensional (3D) structure of the cCNF coating promoted cell survival. The c+aCNF construct proved to combine benefits from both types of CNFs, which means that the c+aCNF cell carrier is a promising candidate for further application in skin tissue engineering.

1. INTRODUCTION

Cellulose is frequently used in the production of biomaterials as a scaffold material to carry other molecules, supporting cell adhesion and promoting cell proliferation. Cellulose is an affordable biocompatible material that can be processed into cellulose nanofibrils (CNFs) and nanocellulose-based matrices with precisely controlled physical and chemical properties. Wood-derived CNFs are manufactured from wood pulp using mechanical techniques alone or in combination with chemical and enzyme-assisted pretreatments. The physicochemical parameters of CNFs, such as swelling, are affected by hydrogen bonding and can be tuned by modifying the CNF dimensions or the charge density. The common length of CNFs is within the micrometer range, whereas the width of CNFs is in the nanometer range, which mimics the dimensions of extracellular matrix (ECM) components, such as collagen fibrils. Moreover, CNFs form multiple types of solids, such as hydrogels, aerogels, and films with the stiffness range of the dermis and epidermis. These materials absorb significant quantities of water, which is a desired property of wound dressings. The surface properties of wound dressings also have an effect on cellular behavior. The CNF surface has a weak negative charge in an aqueous solution in the unmodified form, which is not optimal for the growth of mammalian cells. To enhance the cell–surface interactions, the CNF surface can be chemically modified to adjust the hydrophilicity or the surface charge, depending on the requirements of different cell types.

It has been reported that the modifications of CNFs with cationic or anionic functional groups promoted cell adhesion, proliferation, cytocompatibility, cell growth directionality, water solubility, and bonding of other bioactive molecules, such as collagen and other peptides and proteins. The beneficial effect of CNF hydrogels is especially emphasized in three-dimensional (3D) cell cultivation. The application of CNFs as a wound dressing has also recently reached the clinical evaluation phase, and it...
has been examined as a promising scaffold under in vitro conditions.9,17,18

There are two common chemical modifications that change the charge of the cellulose chain: (1) grafting of anionic 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) oxidation19

and (2) grafting of cationic glycidyltrimethylammonium chloride (GTMAC).6 The benefit of these modifications is in adjusting the desired surface properties of CNFs, e.g., for controlled adsorption of bioactive molecules, which can be further stabilized by cross-linking, driven by chemical functional groups introduced into the cellulose molecule.7 TEMPO-mediated oxidation also allows low-energy mechanical disintegration of oxidized fibers.19 TEMPO-oxidized anionic CNFs (aCNFs) promote cell adhesion and growth,7 GTMAC-modified cationic CNFs (cCNFs) effectively adsorb negatively charged compounds,10,20 including cell adhesion-mediating proteins,6,21 mediate the binding and the release of hydrophobic drugs,22 have an antimicrobial effect,23 and facilitate cell attachment through electrostatic interactions.6 The attachment of anchorage-dependent cells to CNFs is essential for a newly developed skin cell carrier. The cell adhesion can be regulated by modulating the roughness and the stiffness,24,25 the surface chemistry,26,27 the wettability,28 or the electrostatic forces of the substrate.29

The present work enhances knowledge about the behavior of cells on promising charged CNF coatings of commercial, economical, and environmentally friendly cellulose meshes. To find an appropriate cell carrier for engineering and wound healing applications, 3D and two-dimensional (2D) coatings of cationic (cCNF), anionic (aCNF), and combined (c+aCNF) nanocellulose were deposited on commercially available cellulose meshes. Since the proposed materials are to be used for skin applications, the CNF-coated meshes were tested in vitro with normal human dermal fibroblasts (NHDFs) and with hypodermal human adipose-tissue-derived stem cells (ADSCs).

2. MATERIALS AND METHODS

2.1. Production of Cellulose Nanofibrils. The CNF production and characterization were performed according to the study by Skogberg et al.26 Both anionic and cationic CNF grades were produced from once-dried bleached birch kraft pulp. Anionic CNFs (aCNFs) were produced using TEMPO-mediated oxidation, as described by Saito et al.,28 see Supporting Methods (S1.1). Cationic CNFs (cCNFs) were produced by introducing a positive charge using 2,3-epoxypropyl trimethylammonium chloride (EPTMAC; Raisacat, Chimagate, Lapua, Finland); the protocol is described in detail in our earlier study.7 CNF materials were received from VTT Technical Research Centre (Espoo, Finland).

2.2. Preparation of CNF-Coated Meshes. PurCotton highly pure spunlace nonwoven cotton fabric (Winner Industrial Park, Shenzhen, China) was cut into 1.5 × 1.5 cm² samples (further referred to as “noncoated meshes”). The meshes were fixed into CellCrown inserts (Scaldex Ltd., Tampere, Finland), which were inserted into 24-well cell culture plates (TPP, Trasadingen, Switzerland).

The CNF gels were diluted to a 0.15% (w/v) solution in Milli-Q water, sonicated for 2 min at 20% amplitude, and centrifuged at 10 000 g for 60 min.27 The CNF-coated meshes were prepared using either 150 or 600 μL of cCNF and aCNF supernatants (further referred to as “c150, c600, a150, a600”) and by a combination of these supernatants (c+a), as described in Supporting Methods (S1.2). A pristine noncoated mesh was used as the control. The CNF-coated samples were dried for 24 h in a laboratory dryer (Binder, Tuttingen, Germany) at 50 °C (Scheme 1). The sterilization included UV-C irradiation of both sides of the sample in a flow box for 20 min. The samples were sterilized twice in inserts in a sterile Petri dish before the CNF coatings were prepared and then after the CNF coatings had been dried.

2.3. Characterization of the Noncoated and CNF-Coated Meshes. The CNF-coated meshes were characterized by scanning electron microscopy (SEM; Section 2.3.1) and atomic force microscopy (AFM; Section 2.3.3). Noncoated meshes were also imaged by microCT (S1.3). In addition, the physicochemical properties, i.e., the wettability (S1.4), the swelling ratio (Section 2.3.2), and the surface stiffness (Section 2.3.3), were studied.

2.3.1. Topography of CNF-Coated Meshes Using SEM. Front and side views of the CNF-coated meshes (c150, c600, a150, a600, and c+a) and noncoated meshes were acquired using SEM (ULTRAplus, Carl Zeiss, Oberkochen, Germany). The samples were attached to aluminium SEM stubs using a carbon tape and carbon-coated to avoid charging during the SEM studies. The front view was scanned from the surface of the CNF-coated or noncoated meshes, while the side view was scanned from the cut edges.

2.3.2. Swelling Ratio Measurements. The swelling ratio was measured either on a600, c600, and c+aCNF-coated and noncoated meshes (n = 3) or the corresponding glass coverslips (600 μL, n = 3). The initial dry weight (W0) was measured before the samples were immersed in deionized water (dH2O) or in Dulbecco’s modified Eagle medium (DMEM) at 37 °C. The swollen samples were weighed (Wt) at two time points: after 20 min and after 20 h. After being weighed at time point 1 (20 min), the samples were returned into dH2O or DMEM and incubated at 37 °C. The water uptake, subsequently referred to as the swelling ratio (SR), was determined as SR = (Wt − W0)/W0. The data was presented as the arithmetic mean ± standard deviation (SD) from three parallel samples for each experimental group.

2.3.3. Surface Mapping and Characterization of Mechanical Properties Using AFM. AFM data were acquired only for the samples coated with c600, a600, and c+a. The coatings were prepared by gradual application of 600 μL of CNF solution on both sides of the cellulose mesh. The first step is described in Supporting Methods (S1.2). 3D printed tubes fitted on the CellCrown inserts from the outer side were utilized for applying the CNF solution in the second
step. Young’s modulus and the surface arithmetic average roughness (R_a) were determined on the coating from the outer side of the samples. The samples in the inserts were mounted into a custom holder and mapped using an Olympus IX 81 camera (Japan) linked to a JPK NanoWizzard 3 AFM microscope (JPK, Berlin, Germany).

Roughness maps of the dry samples were mapped in the hybrid acquisition mode (Quantitative Imaging mode, QI) with an SNL-10A probe (tip radius 2 nm, cantilever spring constant 0.361 N/m, sensitivity 15.4 nm/V). Bruker AFM Probes, Billerica, MA). QI images were acquired. The dry samples were further examined for their mechanical properties in the same setup as the roughness mapping but with an MFP-12000-10 probe (tip radius 8 nm, cantilever spring constant 5.05 N/m, sensitivity 13.1 nm/V; Bruker AFM Probes, Billerica, MA); for details, see Supporting Methods (S1.5).

The mechanical properties of the wet samples were probed at time points of 20 min and 17 h after immersion in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich Co., St. Louis, MO, Cat. no. D2920). In addition, the probe was replaced by a C7-pp-GONT-60 colloidal probe (tip diameter 6.62 nm, cantilever spring constant 0.391 N/m, sensitivity 45.23 nm/V; NanoAndMore, Wetzlar, Germany); see Supporting Methods (S1.5).

All measurements were preceded by a calibration process. Two parallel samples (eight measurements in total) were used for each experimental group, and the data were presented as the median (Mdn) and the interquartile range (IQR).

### 2.4. Evaluation of Cell Behavior on CNF-Coated Meshes

Two cell types (Section 2.4.1) were cultured on meshes coated with each of the CNF coatings and on the noncoated meshes (Section 2.4.2) to compare the dependence of the cell behavior on the parameters of the sample. The proliferation (Section 2.4.3), the morphology (Section 2.4.4), and the protein-mediated adhesion (Sections 2.4.5, 2.4.6, and S1.12) of the cells were evaluated.

#### 2.4.1. Cell Models and Culture Conditions

Neonatal normal human dermal fibroblasts (NHDFs; Lonza, Basel, Switzerland, Cat. no. CC-2509) were cultured in the DMEM medium with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, Cat. no. 10270-106) and 40 μg/mL gentamicin (LEK, Ljubljana, Slovenia). Adipose-tissue-derived stem cells (ADSCs) were isolated from liposaplates after donors’ confirmed written informed consent had been obtained, in compliance with the Declaration of Helsinki, and under ethical approval by the Ethics Committee at Na Bulovce Hospital in Prague. The isolation procedure for the ADSCs was performed according to Estes and co-authors, with slight modifications as previously described. The pooled ADSCs (for details, see Supporting Methods S1.6) were cultured in the DMEM medium and supplemented with 10% FBS, 40 μg/mL gentamicin, and 10 ng/mL recombinant human basic fibroblast growth factor (PFGF2; GenScript, Piscataway, NJ).

#### 2.4.2. Cultivation of Cells on CNF-Coated Meshes

The CNF-coated and noncoated meshes, fixed into CellCrown inserts and placed into 24-well cell culture plates (see above), were seeded with NHDFs and ADSCs in passage 3 at a density of 20,000 cells/insert (i.e., approx. 25,000 cells/cm²) in 1 mL/well of the cell culture medium. An amount of 0.5 mL/well was added after 2 h of cultivation into the final volume of 1.5 mL/well. The cells were cultivated for 7 days in a cell incubator at 37 °C in a humidified air atmosphere with 5% CO₂. The behavior of the cells was evaluated in three time intervals (days 1, 3, and 7), and the culture wells were 24-well polystyrene plates (PS) were used as a control material.

#### 2.4.3. Metabolic Activity of the Cells on CNF-Coated Meshes (Resazurin Assay)

The level of metabolic activity of the NHDFs and ADSCs on cellulose meshes with all CNF coatings was measured as an indirect marker of the cell number in three time intervals (days 1, 3, and 7) using the conversion of resazurin sodium salt (Sigma-Aldrich Co., St. Louis, MO, Cat. no. R7017) into resorufin by mitochondrial enzymes (for details, see Supporting Methods S1.7). Four parallel samples were used for each experimental group and each time interval. The data were presented as the arithmetic mean ± standard deviation and were used for constructing growth plots to view the overall growth dynamics of the cells.

#### 2.4.4. Fluorescence Staining and SEM Imaging of the Cells on CNF-Coated Meshes

The morphology of NHDFs and ADSCs seeded on the cellulose meshes with all CNF coatings was visualized in three time intervals (days 1, 3, and 7) by staining filamentous actin (F-actin) and vinculin, an important protein of focal adhesion plaques. Vinculin was stained to indicate the level of specific cell-mediated cell adhesion and cell spreading. The detailed staining protocol and the imaging setup are presented in Supporting Methods (S1.8).

The morphology of the cells on the CNF-coated and noncoated meshes was further assessed by SEM on day 3 after cell seeding. The dehydrated samples, see Supporting Methods (S1.8) for details, were fixed on aluminum stubs using a carbon tape, followed by gold coating.

#### 2.4.5. Protein Adsorption on CNF-Coated Meshes (Pierce BCA Protein Assay Kit)

The investigated materials were preadsorbed with proteins derived from blood serum—FBS or bovine serum albumin (BSA)—which modulate the cell adhesion. The total amount of these proteins adsorbed on the CNF-coated (c600, a600, c+a) and noncoated meshes was evaluated by the Pierce BCA Protein Assay Kit (Pierce BCA Protein Assay, Rockford, IL); for details, see Supporting Methods (S1.9). Four parallel samples (eight measurements in total) were used for each data point. The data were presented as the arithmetic mean ± standard deviation of the adsorbed proteins (mg/sample), calculated from the BSA calibration curve according to the manufacturer’s protocol. The data were further expressed as the ratio of the adsorbed proteins (mg/sample) to the concentration of the total proteins in 1.5 mL of added FBS and BSA solutions and given as a percentage.

#### 2.4.6. Cell Adhesion on cCNF Coatings Preadsorbed with Proteins

To explain the cell behavior on cCNF coatings, meshes (S1.10) and glass coverslips (S1.11) with cCNF coatings were used for evaluating the dependence of the cell adhesion on the composition of the preadsorbed proteins. The initial cell adhesion on the c600 coatings was observed by fluorescence staining of the cells, as described above (Section 2.4.4). The treated group of cCNF coatings was preadsorbed with proteins for 2 h before cell seeding, while the cCNF coating control group was seeded with cells and proteins simultaneously (Scheme S1).

#### 2.5. Statistical Analysis

The statistical significance of the data measured by AFM was evaluated using the nonparametric analysis of variance (Kruskal–Wallis), with Tukey’s posthoc test for pairwise comparison. Values of p ≤ 0.05 were considered significant. If not stated otherwise, the data postprocessing and statistical testing were performed in Matlab (MathWorks Inc., Natick, MA). The parametric data from the measurements of the swelling ratio, the cell metabolic activity, and the protein adsorption were evaluated using parametric analysis of variance (ANOVA) with Tukey’s posthoc test for pairwise comparison. Values of p ≤ 0.05 were considered significant.

### 3. RESULTS

#### 3.1. Structural Characterization of the Noncoated and CNF-Coated Meshes

According to the MicroCT analysis (Figure S1), the average fiber thickness of the noncoated mesh was 7.2 ± 1.97 μm (max. 16.1 μm), while the average void thickness was 44.3 ± 36.3 μm. The noncoated mesh possessed high porosity of 84.9%.

The topography of the noncoated and CNF-coated meshes was analyzed using SEM (Figure 1). Depending on the volume applied on the surface of the cellulose mesh and on the charge of the CNFs, the CNF solutions either penetrated into the pores of the mesh, mimicking the 3D structure of the mesh, or formed an almost flat layer on top of the mesh (Figure S1). The CNF solutions with a volume of 150 μL (a150, c150) covered the individual fibers of the mesh and filled the pores between them (see the first column in Figures 1 and S1), while a volume of 600 μL (c600, a600) formed a thin film on the
The swelling ratios of the noncoated and c600, a600, and c+aCNFs coated meshes were determined after 20 h of incubation period. The swelling ratio was measured in dH2O in the c600 coatings. However, the water uptake by the a600 coatings became softer during incubation in DMEM. The swelling ratio in DMEM was measured (Figure 2B). The measured contact angles are reported in the Supporting Information (Figure S2).

3.2. Swelling Ratios and Contact Angles of CNF Coatings. The swelling ratios of the noncoated and c600, a600, and c+aCNFs coated meshes were determined after 20 h in deionized water (dH2O) and DMEM. The highest swelling ratio was measured in dH2O in the c600 coatings. However, there was no significant difference between CNF-coated and noncoated meshes in DMEM (Figure 2A). Due to the considerable water uptake of the underlying meshes, the CNF coatings were prepared on glass coverslips and the swelling ratio in DMEM was measured (Figure 2B). The swelling ratio of the c600 coatings was significantly higher than those of the a600 and c+a coatings after 20 min in DMEM, but after 20 h, there was no additional water uptake by the c600 coatings (Figure 2B). However, the water uptake by the a600 coatings increased with time, and the values after 20 h were significantly higher ($p = 0.03$) than the values after 20 min (Figure 2B). The swelling ratio of the c+a coatings increased slightly between 20 min and 20 h (Figure 2B). The swelling ratios of the noncoated meshes in DMEM and dH2O were comparable (Figure 2A), and they remained relatively unchanged during the incubation period in DMEM (Figure 2B). The measured contact angles are reported in the Supporting Information (Figure S2).

3.3. Stiffness of the CNF Coatings Measured Using AFM. The average stiffness values (arithmetic mean ± SD) of c600, a600, and c+aCNF-coated meshes in the dry state were $0.572 \pm 0.24$ GPa (Mdn = 0.640), $0.683 \pm 0.45$ GPa (Mdn = 0.538), and $0.315 \pm 0.10$ GPa (Mdn = 0.275), respectively. Although there was a significant difference in coating stiffness between a600 and c+a ($p = 0.034$), the mean Young moduli reached the same order of magnitude, and the confidence interval demonstrated only a negligible difference (Figure 2C).

The average stiffness values of the CNF-coated meshes after wetting in DMEM for 20 min were $121 \pm 16$ kPa (Mdn = 116), $342 \pm 101$ kPa (Mdn = 326), and $241 \pm 83$ kPa (Mdn = 230) (Figure 2D—0 h). Young's moduli in the wet state (Figure 2D) are, on an average, three orders of magnitude lower than that in the dry state (Figure 2C), which indicates considerable softening of the materials after wetting. The c600 coatings were significantly ($p = 0.002$) softer than the a600 coatings, while the stiffness of the c+a coatings was between them after 20 min in DMEM (Figure 2D, 0 h).

The average stiffness values of the CNF-coated meshes after 17 h in DMEM were $131 \pm 37$ kPa (Mdn = 121), $173 \pm 117$ kPa (Mdn = 194), and $219 \pm 174$ kPa (Mdn = 221), respectively (Figure 2D, 17 h). Although the median values of a600 and c+a coatings were higher than the c600 stiffness values, no significant difference between the tested groups was found (Figure 2D, 17 h). There was a significant difference ($p = 0.034$) within the a600 coatings, depending on the time scale, while Young's moduli of the c600 ($p = 0.999$) and c+a ($p = 0.848$) coatings did not differ significantly after 17 h from the situation after 20 min. The stiffness of the a600 coatings decreased with time (Figure 2C), while the water uptake increased with time (Figure 2A), which means that a600 coatings become softer during incubation in DMEM.

3.4. Cell Behavior on CNF-Coated Meshes. 3.4.1. Overall Growth Dynamics of Cells on CNF-Coated Meshes. The growth of NHDFs and ADSCs on the CNF-coated and noncoated meshes was evaluated at three time intervals by measuring the cell metabolic activity using the resazurin assay. The values of the cell metabolic activity—an indicator of the cell number—were used for constructing growth plots to evaluate the overall growth dynamics of the cells within a 1-week period (Figure S3).
The swelling ratio of the noncoated and c600, a600, c+aCNF-coated meshes were determined after 20 h in DMEM (Figure 2). The swelling ratio in DMEM was measured (Figure 2B). The measured contact angles are reported in the Supporting Information (Figure S2). The swelling ratio of CNF-coated meshes after 20 h in dH2O and DMEM (A). The swelling ratio of CNF-coated glass coveslips after 20 min in DMEM and after 20 h in DMEM (B). Arithmetic mean ± SD from three independent samples, ANOVA, Tukey’s method, and statistical significance (p ≤ 0.05). Young’s modulus before wetting with DMEM (dry state; C) and after wetting with DMEM (wet state; 0 h; 17 h; D). Median and interquartile range from eight measurements, Kruskal–Wallis, Tukey’s method, and statistical significance (p ≤ 0.05).

3.4.3. Day 1: Adsorption of Serum-Derived Proteins on CNF-Coated Meshes. To explain the differences in the initial cell attachment and spreading on the CNF-coated meshes on day 1, c600, a600, c+a and noncoated meshes were preadsorbed with serum-derived proteins modulating cell adhesion (Figure 4). When the samples were pretreated with 1.5 mL of 10% FBS in DMEM, a significantly greater amount of proteins was adsorbed on the c600 and c+a coatings than that on the a600 coatings and on the noncoated meshes (Figure 4A). To estimate the adsorption of non-cell-adhesive BSA from 10% FBS, the samples were pretreated with 1.5 mL of 0.25% BSA in DMEM, which theoretically corresponds to the average concentration of BSA in 10% FBS. Interestingly, the absolute amount of adsorbed BSA on the c600 coatings was lower than that of the absolute amount of proteins adsorbed from 10% FBS (Figure 4A). However, the percentage of BSA (15.6%) adsorbed on c600 from the BSA solution was higher than the percentage of proteins (13.1%) adsorbed from 10% FBS (Figure 4B). In contrast, the percentage of BSA (5.5%) adsorbed from the BSA solution on the a600 coatings was lower than that of the percentage of proteins (6.9%) adsorbed from the 10% FBS (Figure 4B). On the c+a coatings, the BSA (12.4%) adsorbed from the BSA solution in a similar proportion to the proteins (12.5%) adsorbed from the 10% FBS (Figure 4B). These results indicate that the c600 and c+a coatings adsorbed more non-cell-adhesive BSA from the FBS than the a600 coatings and noncoated meshes adsorbed. This may explain the lower cell adhesion on the cCNF coatings than that on the cCNFs.
and due to the adsorption of the serum-derived proteins predominantly on the cCNFs (Figure 4A,B), the initial adhesion of cells on preadsorbed serum-derived proteins was studied only on the cCNF coatings. On the cCNF-coated meshes preadsorbed with FBS, the ADSCs and NHDFs adhered in low numbers, and their shape after 24 h was abnormal and nonphysiological (Figure 4C,D). However, when the cells were seeded in DMEM supplemented with FBS on pure cCNF-coated meshes, the adhered ADSCs were almost confluent and were well-spread after 24 h. In contrast, the number of adhered NHDFs remained low, and their morphology was comparable with the NHDFs on the cCNF coatings preadsorbed with FBS. Similar results were observed in BSA, although this protein is nonadhesive for cells. On meshes preadsorbed with BSA, both cell types were unable to spread after 2 h. However, when the cells were seeded on pure cCNF-coated meshes in DMEM supplemented with BSA, the ADSCs adhered in greater numbers than the NHDFs and showed some tendency to spread after 2 h of cultivation (Figure 4C,D).

In addition to the investigation into cCNF-coated meshes, the response of NHDFs and ADSCs to the preadsorbed proteins was investigated on cCNF-coated glass coverslips to eliminate the effect of the underlying mesh and to enable live-cell imaging. The results were basically similar to those obtained on the cCNF-coated meshes, i.e., (1) poor adhesion and spreading of both cell types on glass coverslips preadsorbed with FBS or BSA, (2) almost equally poor adhesion and spreading of NHDFs seeded on pure glass coverslips in a medium supplemented with FBS or BSA, but (3) relatively good adhesion and spreading of ADSCs seeded on glass coverslips in a medium with FBS or BSA (Figure S4).

These results suggest that ADSCs adhere more quickly than NHDFs; that is, mostly before the adsorption of non-cell-adhesive albumin, which is contained in FBS. This assumption was confirmed by live-cell imaging using cell trackers. Simultaneous seeding of NHDFs (red) and ADSCs (green) on a cCNF-coated glass substrate in DMEM with 10% FBS revealed that the attachment and spreading of ADSCs was quicker than the attachment of NHDFs, which were much slower in their attachment and were not able to spread properly (Supporting Video).

3.4.5. Day 3: Proliferation and Morphology of Cells on CNF-Coated Meshes. Measurements of cell metabolic activity

**Figure 3.** Metabolic activity (A, B) and the morphology (C, D) of NHDFs and ADSCs on CNF-coated and noncoated meshes on day 1 after cell seeding. (A, B) Metabolic activity of the cells on CNF-coated and noncoated meshes is displayed as a value relative to the metabolic activity of the cells on polystyrene (PS = 100%; red lines). Arithmetic mean ± SD from eight measurements made on four independent samples, ANOVA, Tukey’s method, and statistical significance (p ≤ 0.05). N, No significant difference in comparison with the noncoated mesh (mesh). (C, D) F-actin in the cell cytoskeleton is stained in red and vinculin is stained in green. A confocal microscope with an objective magnification of 20x. Scale bar = 50 μm.
after 3 days of cultivation (Figure S3A,B) revealed that cCNF-coated meshes (c150, c600) significantly increased the proliferation capacity of both cell types compared to the noncoated meshes (mesh) and the cCNF-coated meshes (c150, c600). The metabolic activity of NHDFs on cCNF-coated meshes (c150, c600) was almost at the same level as on noncoated meshes, and it remained at almost the same level as on day 1 (cf. Figures SSA and 3A). The negative influence of cCNFs on cell behavior started to be visible also in ADSCs (Figure S3). However, the metabolic activity of the ADSCs was still slightly higher on the c150 and c600 coatings than the metabolic activity of the NHDFs (Figure SSA,B). Similarly to day 1 (Figure 3A,B), the metabolic activity of both cell types on the c+aCNF-coated meshes was comparable with the values.
on the control polystyrene (PS = 100%) but was significantly lower than the values on the a600 CNF-coated meshes (Figure S5A,B).

After 3 days of cultivation, the cells were mostly spread, and the morphology and the orientation of both cell types were guided by the surface topography of the CNF-coated meshes. The flat 2D surface of the c600 coatings markedly supported the growth of ADSCs (Figure 5B), while the NHDFs were poorly spread and started to detach from the c600 coatings (Figure 5A). However, this rounded morphology of the NHDFs was slightly improved by the 3D topography of the c150 coatings, where the cells acquired a more physiological spindle-like morphology (Figure 5 and S5C). Similarly, although the number of both cell types was higher on the 2D surfaces of the a600 coatings, more elongated cells were observed on the 3D surface of the a150 coatings. The cells on noncoated meshes were round and were barely attached (Figures 5 and S5C,D).

3.4.6. Day 7: Final Colonization of the CNF-Coated Meshes with Cells. The metabolic activity of ADSCs on all types of tested materials was generally lower than the metabolic activity of the NHDFs (Figure S3). However, the proliferation of both cell types on the cCNF-coated and noncoated meshes was significantly lower than that on the aCNF- and c+aCNF-coated meshes. Despite the overall low growth capacity of the cells on the cCNF coatings, the numbers of both cell types were higher on c150 than those on c600 (Figure S6A,B). On a600 and c+a, both cell types reached confluence and colonized almost the entire surface (Figure S6C,D). On day 7, the cell growth capacity on the 3D surface of a150 was equalized with the cell growth capacity on a600 and on c+a, especially in the case of NHDFs (Figure S6).

The 3D projections of microscopy images of cells on the CNF-coated meshes revealed that the 2D coatings (a600 and c+a) enhanced the proliferation and spreading of both cell types only in the xy directions, while the 3D coatings (a150) supported elongation of the cells on the mesh fibers and between them in all xyz directions (Figure 6). Therefore, the 3D surface of the a150 coatings provided more space for cell elongation and proliferation (Figures 6 and S6). The negative effect of cCNF, mainly of c600 coatings, on cell attachment was manifested by the formation of clusters of ADSCs and spheroids of NHDFs (Figures 6 and S6).

Figure 6. Morphology of NHDFs (A) and ADSCs (B), guided by the topography of the CNF-coated meshes after 7 days of cultivation. 3D projection of microscopy images (front view and side view) of the cells on CNF-coated meshes. F-actin of the cell cytoskeleton is stained in red, and vinculin in the cells is stained in green. A confocal microscope with an objective magnification of 40×.
The interaction of the cells with the material surface topography and the formation of vinculin-containing focal adhesion sites were evaluated from 3D projections of microscopy images (Figure 6). During 1 week of cultivation, the interactions between the cells and the surface topography features were established, and a positive effect of the 3D topography of the a150 coatings and also of the c150 coatings on cell growth was observed (Figures 6 and S6,C,D). The cells detaching from the c600 CNF-coated meshes showed vinculin dispersed throughout the cell without creating any focal adhesions, except for the sphericaloid structures, where vinculin seems to be located in the cell—cell contacts. On the c150 CNF-coated meshes, vinculin was more visible in the cells, but only a few barely detected focal adhesions were observed (Figure 6). On the aCNF-coated meshes, the cells were spread in all three dimensions and grew along the directions of the mesh fibers, and focal adhesions were clearly visible (see a150 in Figure 6). On the predominantly 2D surface of the a600 coatings, a higher vinculin signal was observed in the cells that were migrated upward to the top of the protruding aCNF-coated mesh fibers (see a600—arrows in Figure 6).

4. DISCUSSION

4.1. Properties of CNF-Based Coatings. In this study, we prepared nanocellulose-based coatings on a microfibrous cellulose mesh to improve its properties as a cell carrier in skin tissue engineering and wound healing applications. Based on the amount of the CNF solutions that was applied, two different coating microtopographies were formed. A greater volume of the CNF solution (600 μL) resulted in the formation of a flat film-like coating on the surface of the cellulose mesh, while a lower suspension volume (150 μL) resulted in a coating that covered the individual fibers of the mesh. Although the topographies can be regulated by the volume of the solutions, the CNF solution predominantly formed a film-like coating on the surface of the cellulose mesh, while the solution of aCNFs leaked into the mesh pores more easily and predominantly covered the individual mesh fibers (Figures 1 and S1). This behavior can be explained by the presence of larger fibrils in the CNF solution than in the aCNF solution. The larger CNF fibrils were accumulated on top of the mesh fibers and prevented further penetration of the CNF solution into the mesh pores, which resulted in a thin film on the mesh surface. This phenomenon was utilized in the preparation of the c+aCNF coatings, where the CNF solution was first applied on the mesh followed by the application of aCNF and then by mixing of the two solutions. The larger CNF fibrils blocked the pores and prevented penetration of the aCNFs. The aCNFs formed ionic cross-linking with the oppositely charged sidechains of the CNFs, as the trimethylammonium (−N(CH3)3)+ group of CNFs can form ionic bonding with carbonyl (−COO−) groups of aCNFs (Fig. 5).

To describe the functionality of the CNF coating at the cell perception level, the surface roughness of the CNF coating was measured. The roughnesses of the c600 (Rz ~ 9.04 nm) and a600 (Rz ~ 10.2 nm) 2D coatings were similar (Figures 1 and S1). The greater variation in the roughness of c600 is probably due to the locally distributed larger fibers present in the c600 coating. The greater roughness of c+a (Rz ~ 53.76 nm) could be due to strong ionic cross-linking between the anionic −COO− and cationic −N(CH3)3+ groups that probably formed the local aggregation of the nanofibrils.

The different surface chemistries of the CNF coatings influence the wettability and the water uptake, which results in the different swelling properties and the different softening dynamics of the coatings. The more hydrophilic surface of aCNFs presents −COO− and hydroxyl (−OH) functional groups that enable hydrogen bonding. Thus, the aCNF coating binds more water on the surface, resulting in a lower contact angle (31°) with water than that on the surface of the CNFs, which contains not only hydrophilic −OH groups but also more hydrophobic methyl (−CH3) groups. The −CH3 groups do not form hydrogen bonds. This renders the CNF surface more hydrophobic, resulting in a higher contact angle on CNFs (65°) with water. The hydrogen-bonding capacity may also influence the penetration of the fibrils into the mesh pores, as discussed above. The cellulose mesh has more hydrogen-bonding sites for −COO− and −OH groups of aCNFs than those for the −CH3 of the CNFs. HPTMA functionalization of the −OH group in C2 of the CNF cellulose backbone resulted in a larger functionalized moiety than that in the case of the smaller COO−-functionalized −OH group in C6 of aCNFs. Thus, the CNFs form a more branched and snagoer structure (see the structures in Chaker and Boufi31) which enables CNFs to take up more water than aCNFs. The positively charged −N(CH3)3+ groups interact with dipolar substituents,37 enabling solvation even though the −CH3 groups make the CNFs more hydrophobic.

The swelling ratio and the changes in surface stiffness were measured with time. The surface stiffness of all wetted CNF coatings was dramatically reduced in comparison with the coatings in a dry state. The greater stiffness of the aCNF coatings in comparison with that of the CNF coatings at the beginning can be explained by the capacity of oxidized nanofibrils to build up a strong network held together by hydrogen bonding,33 while the presence of the 2-HPTMA chloride moiety of the CNFs reduces hydrogen bonding and thus weakens the cohesion of the network.33,34 The softening of the CNFs with time could also be due to displacement of the hydrogen bonding between the −COO− and −OH groups by the hydrogen bonding between the −COO− and H2O molecules. In addition, the charged groups of CNFs should also increase the hydrophilicity of the material. It has been reported that TEMPO oxidation increases the negative charge, resulting in electrostatic repulsion between the fibrils in a wet state; thus, the high negative charge of TEMPO-oxidized CNFs causes low water resistance and high swelling.33 The surface charges of our aCNF and CNF coatings are probably similar to the reported ζ-potential values for the corresponding grades of CNFs, which were ~−69.5 mV (for aCNFs) and ~+1 mV (for CNFs).33,39 The negative surface charge, in combination with hydrogen bonding to the water molecules, could explain the swelling and softening of aCNF coatings over time, while the branched sponge-like structure of −N(CH3)3+ groups could contribute to the higher total water uptake of CNF coatings. The swelling of CNFs decreased slightly, while the mean stiffness increased slightly. This is particularly apparent during the immersion of CNFs in DMEM. The ions present in DMEM may enable ionic cross-linking to some extent, which reduces the water uptake and increases the strength of the material, which was observed for CNFs over time. In summary, the general trend in stiffness follows the general trend of the swelling of the coatings. There was a significant difference in swelling of c600 between water and...
DMEM (Figure 2A). Unlike the deionized water used in our experiments, DMEM contains a variety of ions that can interact with the positively charged moieties of c600, and subsequently, fewer water molecules are occupied around these sites, resulting in less water uptake than that in the case of the samples soaked in deionized water (Figure 2A).

The microtopographical structure of the c+a combination is similar to that of the cCNFs, while the contact angle, swelling, and stiffness properties more closely resemble those measured on the aCNF coatings. The roughness of the c+aCNF coating differs from the roughness of both cCNFs and aCNFs. The values of contact angle and the swelling of c+aCNFs are closer to the values of aCNFs than to the values of cCNFs. This is probably due to strong ionic cross-linking between c+aCNFs, which prevents the water from penetrating into the structure. However, the c+aCNF coating contains more hydrophilic hydrogen-bonding groups than the cCNF coatings, which may also have an effect on the swelling and stiffness. The stiffness of the c+aCNF coating did not decrease over time, as was observed with the aCNF coatings. This can be explained by strong ionic cross-linking, which can reduce the interactions of hydrogen-bonding groups with water molecules. The c+aCNF combination, therefore, offers an interesting set of properties, which might be further adjusted to create not only film-like coatings for cell cultivation but also 3D cross-linked CNF gels for cell embedding and for 3D printing.

4.2. Cell Behavior Influenced by the Properties of CNF Coatings. A comparison was made between two coating microtopographies—the 2D film-like topography and the 3D coating topography—for differences in cell—material interactions. Our results suggest that the flat substrates of c600, a600, and c+a, where the cells formed monolayers, can be suitable for cell sheet technology. This technology enables the creation of self-standing, mechanically resistant, continuous cell sheets that can be replanted on other substrates, such as tissue-engineered skin constructs in vitro or wound beds in vivo. The cell sheets can be released from cellulose substrates by cellulase enzymes, which are not cytotoxic. In addition, unlike proteolytic enzymes such as trypsin or collagenase, they preserve the cell-to-cell connections and the ECM proteins within the sheets. The 3D substrates of c150 and a150, where the cells penetrated into the material, are also promising for tissue engineering and for future clinical applications. 3D substrates made of a CNF-coated cellulose mesh might provide sufficient space for the cells for in vitro long-term cell cultivation and might allow for better diffusion of nutrients than the widely used hydrogels.11

Not only the microtopography of the material but also the cell—material interactions can also be modulated by the elasticity, roughness, and surface chemistry of the material. The elasticity of natural tissues varies from 0.2 to 1 kPa for soft tissues, such as brain or fat, via medium-soft tissues such as skin (10–30 kPa), to hard tissues of more than 103 kPa, e.g., for bone.32,43 The stiffness of our CNF coatings is close to the stiffness of native tissues such as fibrotic tissue (100 kPa), cartilage, and tendon (approaching 103 kPa).25,42 By comparison, Kummala et al. introduced 0.1 mm thick CNF films with Young’s modulus between 1 and 60 kPa.3 Although the CNF substrates used in this study were much softer than the conventional cell culture substrates (GPa),42 they can be considered as relatively stiff substrates at the cell perception level. For example, Achterberg et al. determined Young’s modulus of native human dermis using AFM between 0.1 and 10 kPa, depending on the location, which is at least 10 times softer than our CNF substrates.44

Not only the stiffness but also the roughness is important at the cell perception level, especially for initial cell attachment, because the cell focal adhesions are in the nanoscale range from 2 to 200 nm.45 Alterations in material surface roughness can influence the adhesion and further proliferation of fibroblasts, as described by Bourkoula et al., and also the spreading and differentiation of mesenchymal stem cells, as observed by Hou et al.46,47 While the aCNF and cCNF coatings showed relatively low roughness, the c+aCNF combination showed a rougher surface (see Rq ~ 55.76 nm in Figure 1), which corresponds to the least rough surface measured by Hou et al.47 These authors revealed that the substrates with the lowest studied roughness (Rq ~ 31.49 nm) supported cell adhesion and spreading, but the cell—substrate tension and osteogenesis were only moderate in comparison with the substrates with intermediate roughness (Rq ~ 183.16 nm).47

Although the studied CNF surfaces differ in many parameters, including the microtopography, roughness, swelling ratio, and Young’s modulus, we assume that the main cell adhesion-modulating factor is the surface chemistry of CNFs, which governs the surface charge and wettability. The surface chemistry can determine the composition of proteins adsorbed on CNF surfaces and the speed with which various serum proteins are adsorbed and the cells are adhered.24 It is generally known that cells adhere to material substrates through the proteins adsorbed to the material surface from the biological fluids. Adhesion-mediating proteins, such as fibronectin, vitronectin, collagen, and laminin, contain specific amino acid sequences (e.g., RGD) that can be recognized by integrin receptors on cells, while albumin is nonadhesive for cells due to the lack of these specific adhesion sequences in its molecule. However, albumin can improve the geometric conformation of the cell adhesion-mediating proteins for binding by cell adhesion receptors.25 Based on the Vroman effect—i.e., the dynamics with which proteins adsorb and interchange in time—we can assume that the NHDFs and ADSCs adhered to a layer of proteins that differ in their composition.48

We have demonstrated that cCNFs with positively charged –N(CH3)3+ tails adsorb more proteins, predominantly BSA, than aCNFs with negatively charged –COO functional groups (Figure 4). Similar results were achieved by Attwood et al.40 with well-defined positively charged –N(CH3)3+ and –COO−-terminated self-assembled monolayers (SAMs) and also by Courtenay et al., who quantified the proteins adsorbed from FBS, specifically BSA, on positively charged GTMAC-modified cCNF scaffolds.49,50 They revealed increasing protein adsorption with increased cationization. The greater affinity of BSA to positively charged surfaces could be due to the negative character of the BSA at the physiological pH of DMEM.51,52 We could expect that the negatively charged proteins in FBS, such as fibronectin, vitronectin, and BSA, have the same electrostatic affinity to the positively charged surfaces at the physiological pH of DMEM due to their similar theoretical isoelectric points.53 However, the concentration of BSA at a level between 35 and 50 mg/mL in FBS is 100–1000 times higher than the concentration of other proteins,26,27 which makes BSA the most abundant adsorbed protein on positively charged substrates. Furthermore, Hoshiba et al.56 revealed that the absolute amount of adsorbed proteins on chemically
modified charged methacrylates (MA) is less important than the protein composition of the most superficial layer. In their study, positively charged \( -\text{N}^+\text{(CH}_3\text{)\text}_3^- \)-terminated MA adsorbed more proteins from FBS than were absorbed by negatively charged \(-\text{COO}^-\)-terminated MA. However, the most superficial layer of \(-\text{COO}^-\)-terminated MA contained more cell adhesion-mediating proteins, mainly vitronectin, and also fibronectin in its more suitable conformation for cell adhesion.\(^{26}\) In general, the positive effect of negatively charged \(-\text{COO}^-\) termination on cell adhesion, spreading, and proliferation of various substrates has been confirmed by many other research groups.\(^{6,26,53}\)

The abundant yields of BSA on the surface of the cCNF-coated meshes probably reduced the amount of attached NHDFs, but it did not influence the attachment of the ADSCs. Based on Courtenay et al.’s work, we hypothesize that the ADSCs were attracted by the positive charge on the surface of the cCNFs, and their attachment can be classified as protein-independent electrostatically mediated adhesion.\(^{68}\) Unlike Courtenay et al., we observed this phenomenon with ADSCs not only in a serum-free medium but also in a serum-containing medium (Figure S4 and the Supporting Video).\(^{8}\) This could be due to a higher adhesion speed of ADSCs than that of NHDFs, which enabled them to adhere before the adsorption of BSA took place. This was clearly visible especially on the ADSCs that were not able to adhere to the cCNF coatings, which were preadsorbed with FBS or nonadhesive BSA (Figure 4). Although ADSCs adhered to the cCNF coatings in large numbers, their cell adhesion forces were rather weak, as was manifested by the suppression of proliferation and by the formation of clusters and spheroids. Courtenay et al. reported similar findings, revealing that there was greater cell adhesion on cationic nanocellulose than on anionic nanocellulose in the serum-free medium, while the cells in the serum-containing medium responded in the opposite way.\(^{6}\) This confirms that the cells in a serum-free medium adhered to positively charged substrates via integrin-independent electrostatic interactions, while cell adhesion was suppressed in the serum-containing medium by the adsorption of the proteins in an inappropriate spectrum and conformation. Negatively charged substrates therefore seem to be more suitable for cells due to the presence of stronger protein-mediated integrin-dependent adhesions and also weak electrostatic cell–material interactions.\(^{26,53}\) In addition, this specific integrin-mediated cell adhesion is capable of delivering appropriate signals to cells, ensuring the viability, proliferation, and other functions of the cells.

The adsorption of proteins and further cell adhesion is also mediated by the hydrophilicity or hydrophobicity of the surfaces.\(^{27,26,53}\) Although both CNF coatings were expected to be hydrophilic due to the charged \(-\text{COO}^-\) and \(-\text{N}^+\text{(CH}_3\text{)\text}_3^- \) functional groups,\(^{36}\) the cCNFs (contact angle 65°) were more hydrophobic than the aCNFs (contact angle 31°).\(^{36}\) The presence of neutral \(-\text{CH}_3\) groups on the surface of cCNFs probably reduced the hydrophilicity, which made the surface less attractive than the surface of aCNFs for the cells in a serum-containing medium. Similar results were achieved by McClary et al. and Faucheux et al. with \(-\text{CH}_3\) and \(-\text{COO}^-\)-terminated SAMs.\(^{53,54}\) In addition, Arima and Iwata\(^{37}\) described how moderately hydrophilic SAMs enhanced cell adhesion even though they were preadsorbed with BSA, while hydrophobic SAMs preadsorbed with BSA inhibited cell adhesion. This was due to the strongly adsorbed BSA on hydrophobic SAMs, which cannot be replaced by cell adhesion-mediating proteins as effectively as on hydrophilic SAMs, where the proteins interchanged due to the Vroman effect.\(^{27,44}\) We could assume that both aCNFs and c+aCNFs were suitable for cell adhesion and growth due to their hydrophilic surface character. Although the level of adsorbed BSA on c+aCNFs was comparable to the level of less hydrophilic cCNFs (Figure 4), the hydrophilic surface character of c+aCNFs probably enabled BSA to be replaced by the cell adhesion-mediating proteins in the most superficial layer of the adsorbed proteins. However, the less adhesive cCNF coatings for cells can also occupy an important place in tissue engineering. As indicated by Figures 6 and S6, these surfaces can be used for generating three-dimensional multicellular spheroids, similar to the albumin-coated surfaces in a study by Okuyama et al.\(^{55}\)

5. CONCLUSIONS AND FURTHER PERSPECTIVES

In this study, we have developed novel electroactive coatings on a cellulose mesh based on cationic nanofibris (cCNFs) or based on anionic cellulose nanofibrils (aCNFs) or based on a 1:1 mixture of both types of nanofibrils (c+aCNFs). When seeded with normal human dermal fibroblasts (NHDFs) or human adipose-tissue-derived stem cells (ADSCs), the negatively charged aCNFs, and also the mixture of c+aCNFs, appeared to provide better substrates for cell adhesion and growth than the positively charged cCNFs. This was demonstrated mainly with NHDFs. The most likely explanation for this finding is that the positively charged cCNFs were more hydrophobic and they preferentially adsorbed albumin, which is nonadhesive for cells. However, negatively charged aCNFs and combined c+aCNFs were hydrophilic, and they adsorbed more serum proteins mediating cell adhesion, such as vitronectin and fibronectin. In addition, cCNFs attracted the cells via electrostatic forces, and this non-integrin-mediated cell adhesion is less efficient in maintaining the viability and the growth activity of cells. Nevertheless, all three types of CNF coatings can be utilized in specific biomedical applications, e.g., in skin tissue replacement, using cell sheet technology in the case of aCNFs and c+aCNFs, and the generation of cell spheroids, in the case of cCNFs. In addition, c+aCNFs provide an interesting combination of the properties of cCNFs and aCNFs: the microtopographical structure is similar to that of cCNFs, while the contact angle, swelling, and stiffness properties more closely resemble the values for aCNF coatings. In the future, the properties of the c+aCNF combination can be further adjusted to create not only film-like coatings for cell cultivation but also 3D cross-linked CNF gels for cell embedding and 3D printing.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c01097.

Supporting methods and figures (PDF)

Adhesion and spreading of NHDFs (red tracker) and ADSCs (green tracker) in coculture on cCNF-coated glass (phase contrast) during the first 22 h after cell seeding (Supporting Video) (AVI)
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Author Contributions
J.P. and A.S. contributed equally to this work. The manuscript was written through the contribution of all authors. All authors have approved the final version of the manuscript. J.P. and A.S. planned, performed, and analyzed the experimental work, with the exception of the microCT imaging and analysis (M. Hannula) and the AFM scanning and analysis (D.H.). A.B., M.Z., and M. Tomkova helped with the cell experiments and microscopy imaging. M. Travnickova and J.P. performed the ADSC isolations. M. Honkanen performed SEM scanning. P.L. contributed to the CNF preparation. P.K. and L.B. contributed to the conception of the study, coordination of the work, and structuring and editing the manuscript.

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Notes
The authors declare no competing financial interest.

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Author Contributions

- B. B. C., D. H. M., and M. T. helped with the cell experiments, and H. H. performed the AFM scanning and analysis. A. B., P. L., M. T., and M. T. helped with the cell experiments, and H. H. performed the AFM scanning and analysis.

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Self-assembled cellulose nanofiber-carbon nanotube nanocomposite films with anisotropic conductivity


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Self-assembled cellulose nanofiber–carbon nanotube nanocomposite films with anisotropic conductivity†

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In this study, a nanocellulose-based material showing anisotropic conductivity is introduced. The material has up to 1000 times higher conductivity along the dry-line boundary direction than along the radial direction. In addition to the material itself, the method to produce the material is novel and is based on the alignment of cationic cellulose nanofibers (c-CNFs) along the dry-line boundary of an evaporating droplet composed of c-CNFs in two forms and conductive multi-walled carbon nanotubes (MWCNTs). On the one hand, c-CNFs are used as a dispersant of MWCNTs, and on the other hand they are used as an additional suspension element to create the desired anisotropy. When the suspended c-CNF is left out, and the nanocomposite film is manufactured using the high energy sonicated c-CNF/MWCNT dispersion only, conductive anisotropy is not present but evenly conducting nanocomposite films are obtained. Therefore, we suggest that suspending additional c-CNFs in the c-CNF/MWCNT dispersion results in nanocomposite films with anisotropic conductivity. This is a new way to obtain nanocomposite films with substantial anisotropic conductivity.

Introduction

There is an increasing demand for thinner, cheaper, lighter, and more eco-friendly electrically conductive films and composites1 with anisotropic properties2,3 for applications including optics, sensors, actuators, aerogels, mechanical reinforcement of advanced materials, and growth guiding substrates including hydrogels for biomedical applications.2,3 There are various motivations for material alignment in these applications, such as a need for anisotropic properties in adjusting the stiffness and strength of structural materials; optimizing the reinforcement efficiency in fiber-reinforced composites; and mimicking the topography and architecture of native tissues in biomedical materials and applications, especially related to cell alignment in vitro.2 The assembled oriented particles with a high aspect ratio generally exhibit anisotropic physical properties. If the particle is also conductive, an anisotropic conductivity may result, which is potentially useful in electronic components, capacitors, sensors, electromechanical actuators, etc. In the assembled structures of high aspect ratio nanomaterials, the differences in the electric current parallel and perpendicular to the orientation results in conductivity anisotropy.4

Cellulose nanofibers (CNFs) are an excellent candidate as a primary ingredient in such composites since they provide an eco-friendly and low-cost material alternative1 with the potential to align and offer anisotropic properties for the resulting composite material.2 CNF-based electrically conducting materials have been produced by coating, blending or doping CNFs with conductive nanoparticles such as carbon nanotubes (CNTs). CNFs act as a matrix and dispersant of the conductive CNT component.5–7 These nanomaterials together provide a combination of their superior properties such as the film forming potential,8 favourable mechanical and chemical properties, colloidal stability, cytocompatibility9–13 and biocompatibility of CNFs,9–11 and high mechanical strength, stiffness, and electrical and thermal conductivity of CNTs.16

Chemically added charged groups influence the physico-chemical properties of CNFs17 and can improve their processa-
bility and performance. Treatment with cations can be performed to render cellulose nanofibers cationic by introducing positive charges on their backbone. Cationic CNFs (c-CNFs) can be used in both high-end applications as nanocomposites and high volume applications, and are gaining increasing attention as a potential novel nanomaterial with tailored properties in more specialized applications. Cationic CNFs have the capability to align along the evaporating droplet boundary line by self-assembly mechanisms. By dispersing carbon nanotubes with c-CNF, there is potential to form aligned structures also in electrically conductive composites. None of the previous studies have used c-CNFs in the dispersion of CNTs. In the current study, the use of cationic CNFs as a dispersing and stabilization agent is reported for the first time and their alignment is studied in nanocomposite films.

Superior CNF properties are often challenging to transfer into optimal macroscale performance. In plants, CNFs are naturally aligned into highly hierarchical structures. However, despite several attempts and extensive research, the realignment of disintegrated and fibrillated CNFs remains challenging. While the alignment of CNFs alone is challenging as such, it is extremely challenging to align CNFs in composites. Another challenge is dispersing CNTs because of their self-aggregation tendency especially in aqueous media, which on the other hand is often a prerequisite for hydrophilic CNFs. While CNT blending in aqueous media is poor due to strong self-aggregation, sonication treatments have been introduced to obtain homogeneous dispersions. Several research groups have reported the ability of CNFs to disperse CNTs in aqueous media using sonication and act as a stabilization agent in the CNF–CNT dispersion; furthermore, a stable dispersion can be obtained without the use of surfactants, which have been commonly used. Chemical modifications of at least one of the nanomaterial components by the introduction of charged groups may enhance the dispersing effect. In addition, repulsive forces may prevent the reagglomeration of the particles in the dispersion state resulting in a more stable dispersion. It has been shown, e.g., that anionic CNFs enhance the dispersion of CNTs.

Sonication is one of the methods used for attaining the dispersion of nanoparticles in aqueous media. It is based on inertial cavitation where imploding cavities, which are known to exist at the boundaries of materials, generate intensive streams of molecules. In sonication, a high energy density is introduced to the CNT–CNT interface, which is high enough to cause initial separation of the nanotubes by breaking noncovalent forces between tubes. However, the used amount of energy should be optimized such that only noncovalent forces are overcome and the carbon nanotube backbone remains intact. An overly high sonication energy can cause carbon bond breakage inside individual nanotubes, which deteriorates their ballistically conducting nature. The dispersion quality and functionalization obtained in sonication depend on the used sonication energy, medium, surfactant type, and surfactant amount.

In this study, we present a method to produce self-assembled nanocomposite films with adjustable anisotropic thermal and electrical conductivity. The method uses sonochemical treatment and c-CNFs to disperse multiwall carbon nanotubes (MWCNTs). The resulting dispersion is assembled with the help of additional, suspended c-CNFs, which are based on the previously reported c-CNF alignment along an evaporating boundary line. We also show how the alteration of the composition and pre-treatment of the two nanomaterials enable the fabrication of nanocomposite films with either isotropic or anisotropic properties. Controlled nanomaterial assembly commonly requires more time, high energy consumption, and expensive proprietary technology. The method described in this article is simple, quick, efficient, and safe compared to current attempts to control the assembly of nanomaterials. Such novel nanocomposites could be used as a cell guidance material with cells that benefit from the mechanical improvements offered by MWCNTs or in applications in which anisotropic heat or current conductivity is essential. CNF-based electrically conductive composites have many advantages although many challenges remain, e.g., related to electrical connectivity. In our previous study, we have also shown that MWCNTs can be effectively dispersed with unmodified CNFs with the help of surfactants and form highly conductive nanocomposite films with a relatively low MWCNT concentration. In the present article, we combine the advantages of these observed phenomena to create a totally new methodological concept: c-CNFs are used as an aid to disperse MWCNTs homogeneously to form a stable dispersion, and additional c-CNFs in the dispersion undergo interfacial self-assembly with the high energy sonicated (hes-) hes-c-CNF/MWCNT dispersion, resulting in a novel nanocomposite film with anisotropic properties.

Results and discussion

Theoretical assumption and modelling

In our previous study, we showed c-CNF alignment along an evaporating boundary line. In the present study, we studied if a conducting component (MWCNT) can be added, and still obtain anisotropic film properties. We also have studied dispersing MWCNTs using CNFs and sonication, and thus hypothesize that CNFs used as a dispersant form a stronger complex with MWCNTs, compared to CNFs that are added to the system by more gentle manual mixing. Based on our previous findings, we hypothesize as shown in Fig. 1, that an isotropic conductive film is obtained when water from the hes-c-CNF/MWCNT dispersion is evaporated and an anisotropic film is obtained when water is evaporated from a suspension composed of the hes-c-CNF/MWCNT dispersion and additional c-CNFs. Fig. 1 conceptually depicts the hypothesis for explaining the structural composition and the electrical conductivity of the films fabricated from a dispersion (control) or with suspended free c-CNFs. Sonochemical treatment of c-CNFs and MWCNTs is hypothesized to result in a stable dispersion.
Nanocomposite films formed from this hes-c-CNF/MWCNT dispersion (control samples) are uniformly conducting in all directions (that is, isotropic films), as illustrated by the left-side schematic in Fig. 1(a and b). When free c-CNFs are added to the stable dispersion, a self-assembled structure is formed during evaporation, providing anisotropic electrical properties (that is, assembled films), as depicted in the right-hand-side schematic in Fig. 1(b and c). Fig. 1 shows simulations of electrical properties of isotropic control (a) and anisotropic assembled (d) nanocomposite films using the finite element method (FEM) using COMSOL Multiphysics® Version 5.4 (COMSOL, Inc. USA). Fig. 1e and g show a hypothesized structure at the nanoscale, highlighting the aligning component, which is suspended c-CNFs (Fig. 1g) in the assembled films, while the repeating, alternating structure in Fig. 1g is an oversimplification. The structure is believed to alter at the nanoscale, and the boundaries between aligning c-CNFs and more entangled hes-c-CNF/MWCNTs are not as clear as shown in Fig. 1g. We assume the increasing amount of c-CNFs between the “layers” from the edge towards the center (based on the resistance results presented in Fig. 3). A film manufactured from the hes-c-CNF dispersion exhibits even conduction. The high energy sonication treatment causes c-CNF fibrillation and chopping, resulting in smaller hes-c-CNFs (Fig. 1f) compared to the suspended c-CNFs that have not been high energy sonicated (Fig. 1g). Using thermal imaging and resistance measurement, we aim to confirm this presented Hypothesis of the assembled film with anisotropic conductivity. Furthermore, we performed micro- and nanostructural characterization to provide support for the Hypothesis of an assembled structure and the dispersal effect of c-CNFs. While the exact structure and alignment are challenging to show at the nanoscale, in the next sections we present a variety of results supporting the hypothesis in which the c-CNF alignment drives the formation of anisotropically conductive films.

**Anisotropy characterization: electrical conductivity of nanocomposite films using heat maps**

The heat map results confirm that evaporation of the aqueous medium from the dispersion results in uniform electrically conductive nanocomposite films, while that evaporated from the suspension (S1–S3) formed self-assembled nanocomposite films with anisotropic electrical conductivity. An overview of the differences in conductivity between the isotropic control film and the self-assembled anisotropic films is visible in IR heat maps (Fig. 2). The control film obtained from the sonicated stable homogeneous hes-c-CNF-MWCNT dispersion shows an even temperature across the film when a constant voltage is applied on the film surface through the silver ink contacts, suggesting that the current follows the shortest
Anisotropy characterization: electrical conductivity analysis using resistance measurement

The IR-imaging depicts the anisotropic electrical properties of the self-assembled films at the macroscopic scale. Resistances determined (Fig. 3 and Fig. S1 of ESI†) using the measurement protocol (Fig. 8) confirm the findings obtained with the IR-imaging: the control film shows isotropic behavior, while self-assembled films S1–S3 are electrically anisotropic.

The control films show relatively constant resistance throughout the film, as illustrated in Fig. 3. In contrast to the control films, the self-assembled films S1–S3 show evident anisotropy. The resistance increases along the centre line towards the centre (measurements A and C, Fig. 3), while along the circle it essentially remains constant, measured from both circles 1 and 3 (measurements D and E, respectively, Fig. 3).

In measurement A, the measurement probes are moved along the centre line, maintaining a constant probe distance, depicting an increase in resistance when moving towards the centre, and a subsequent decrease in resistance when moving from the centre towards the edge of the film. The resistance increase is apparent in each measurement towards the centre, although the increase is larger closer to the centre.

Measurement B demonstrates an increase in resistance along the different circular zones, that is, circles when zones are located closer to the centre. Thus, the resistance continually increases even though the probe distance decreases. A difference in the isotropic control film is evident, where the resistance decreases with reduced probe distances, as is expected for an evenly conducting film. Measurement B includes radial line locations that correspond to the location at 180° in measurement D and E, further confirming lower resistances along a circular zone than along the centre line.

In measurement C, one probe remains in location 1 in all measurements, while the other probe is moved along the centre line, that is, the probe distance increases in the subsequent measurements. Measurement C again confirms the difference between the control and assembled films. The resistance in the control film increases slightly with an increasing probe distance, while in the self-assembled films S1–S3, the resistance increases significantly until the centre, with a subsequent decrease after the centre towards the opposite edge of the film. This is in accordance with the Hypothesis, which assumes the current passes through the conducting zones, that is, along the circles in the self-assembled S1–S3 films – instead of the shortest route across the centre, as in the control films.

The resistance measured along circle 1 and along circle 3 in measurements D and E, respectively, is consistent with the Hypothesis. In the control film, the resistance in circle 1 is slightly higher than that in circle 3. This is due to the longer probe distance in circle 1 than in circle 3. Thus, the result is compatible for a uniformly conducting film. Opposite to the control film, the self-assembled films (S1–S3) have a lower resistance in circle 1 than in circle 3, even though the probe distance decreases in circle 3. The result is in accordance with measurement B (Fig. 3), which demonstrated the increase in the resistance along the different circles when zones are located closer to the centre. Opposite results between the control films and films S1–S3 further confirm the Hypothesis of an anisotropic assembly in S1–S3. The increase in resistance of all the measured films (control, S1–S3) when moving along circles 1 or 3 results from the increasing probe distance.

The demonstrated anisotropy is stronger in S2 than in S1 because of a higher concentration of suspended free c-CNFs in S2 than in S1. The concentration of suspended free c-CNFs is
equal in S2 (supernatant) and S3 (supernatant sonicated 625 kJ g$^{-1}$). Even though the concentration of suspended c-CNFs is equal in S2 and S3, the anisotropy is stronger in S3. Suspended c-CNFs are more fibrillated in S3 compared to S2, thus the nanofibrils in S3 are expected to be smaller in diameter.

When comparing resistances on approximately the same distance along circle 1 and along the radial line, that is, probe locations 1 – 45$^\circ$ (measurement D) and 1–4 (measurement C), respectively – the resistance is approximately 10-fold, 100-fold, and 1000-fold higher in the radial line compared to the resistance along circle 1 for S1, S2, and S3, respectively. Comparing the length of the radial line from the edge until the centre of the film – that is, locations 1–5 (measurement C) – and the corresponding distance on circle 1 – that is, locations 1 – 90$^\circ$ (measurement D) – the difference in the resistance along the centre line (from location 1 to location 5) is even higher, approaching 100-fold and 1000-fold in S1 and S2, respectively.

In S3, the resistance in the corresponding locations remains the same as between locations 1 and 4, that is, approximately 1000-fold. In all self-assembled films S1–S3, the highest resistance is found in the middle of the films and is approximately 0.5 MΩ for S1 in locations 4–5, while approaching 10 MΩ and 100 MΩ in S2 and S3, respectively. Composites were subjected to an electric field for at least 11 min as the measurements were repeated three times for each film, to demonstrate the stability of the films. In addition, three parallel films were fabricated and measured in order to show the repeatability of the film manufacture. Parallel and repeat resistance measurement results are summarized in ESI Fig. S1† and the results indicating the stability of the films in the electric field are presented in ESI Fig. S1 and S2.†

**Anisotropy characterization: topographical alignment**

Scanning electron microscopy images were collected at different locations on the film to perform SEM image-based
orientation analysis. Cytospectre software was used for the analysis to obtain the degree and direction of orientation from the assembled films or the lack of orientation from the control films. Using spectral analysis, two different values were obtained to confirm anisotropy from the SEM images of assembled films (Fig. 4). First, we use a parameter called the circular variance (CV) value to show the difference in the degree of orientation between assembled and control films. CV was determined from each image (Fig. 4a, b and h) in order to describe to degree of orientation. A CV value of 1 refers to perfect isotropy, while a value of 0 is perfect anisotropy. While the CV value of the isotropic control film is close to 1 (indicating isotropy), that of assembled films is remarkably lower (indicating more anisotropic topography compared to control films), Fig. 4h.

In addition to the CV value, a mean orientation angle of each image location was determined in order to show the orientation direction along the evaporating boundary line. While CV only describes the degree of orientation – that is, isotropy or anisotropy – the mean orientation can be used to show if the orientation direction is according to our hypothesis; that is, along the evaporating dry-line boundary, and thus along the imaginary circles. For this purpose, the films were scanned on a specific surface location, and the scanning location (Fig. 4c) was compared with the mean orientation angle (Fig. 4e) of the analyzed images (Fig. 4a and b as an

Fig. 4  Low vacuum SEM scans from the surface of the S3 (a) and control (b) films. Scale bar in a and b is 50 µm. Examples of scanning locations (c) and the corresponding mean orientation angles (e) of the assembled films. The mean orientation angles for the control films are not presented, as the circular variance is close to 1 in all scanning locations of the control films (d), so no primary alignment direction is expected. A graphical illustration (f) of the orientation describes the orientation direction in Scanning Point 2 (SEM image in (a)), while isotropy (g) is confirmed for the location seen in SEM image (b). Circular variances of the films S1, S2, S3 and control (h). Circular variance averages (n = 4) or the films S1, S2, S3, and control are 0.76, 0.65, 0.67 and 0.96, respectively. Perfect isotropy corresponds to circular variance value 1, while 0 stands for perfect anisotropy.
example) and the expected orientation direction according to our hypothesis (Fig. 1). The mean orientation is presented only for assembled films, as the control films have no expected orientation according to the analyzed CV values. However, an example of the mean orientation angle for the control sample is shown in Fig. 4b for the scanning location shown in Fig. 4d.

The CV analysis (Fig. 4h) confirmed the expected higher degree of orientation of the assembled films compared to the control films, as discussed below. The degree of anisotropy is greater for assembled films than for control films with more isotropic CV values. As the film also contains isotropic MWCNT arrangement (Fig. S4a, c and f†), CV values are not expected to be close to zero. This is because only the added c-CNFs are expected to align along the dry-line boundary during evaporation. Thus, the average CV values ($n = 4$) of the assembled films S1, S2, and S3 are 0.65–0.76 (Fig. 4h), compared to the previously determined 0.27–0.28 of highly anisotropic pure c-CNF films. The average circular variance ($n = 4$) of the control films is 0.96.

In summary, CV values show a higher degree of anisotropy for assembled films than control films do, while the mean orientation angles in specific locations show orientation along the circle. This indicates orientation along the dry-line boundary during the evaporation of the liquid.

When analyzing the low vacuum SEM surface scans (one example in Fig. S5†), it is evident that the hes-c-CNF fibrillates during sonication, as the larger fiber bundles that can be seen in S1 and S2 (Fig. S5a and b†) are missing in S3 (Fig. S5c†), in which the suspended component was hes-c-CNF. The decreased size of hes-c-CNF compared to c-CNFs is due to the high energy sonication treatment of the former. The sonication energy used in suspended hes-c-CNF in S3 is the same as that used to prepare the hes-c-CNF/MWCNT dispersion. The degree of orientation is significantly higher in the case of S3 (manufactured using hes-c-CNF + hes-c-CNF/MWCNT) compared to the control film (manufactured using hes-c-CNF/MWCNT). This confirms that the alignment is not dependent on the suspended c-CNFs, but the addition of the suspended c-CNFs.

**Nanocomposite film structure supporting the hypothesis**

To confirm the basis behind the observed electric anisotropy, structural characterization of the films and their components was performed using scanning electron microscopy (SEM), (scanning) transmission electron microscopy ([S]TEM) and atomic force microscopy (AFM). According to SEM, [S]TEM, and AFM imaging, MWCNTs were in random orientation in all control and self-assembled (S1–S3) films. Thus, no alignment/orientation of MWCNTs was observed (Fig. 5c and S4a and e†). While it is challenging to observe topographical alignment from the densely packed film surface of low weight element components, SEM images were analyzed using spectral analysis in order to detect possible anisotropy and orientation direction. Spectral analysis (Fig. 4) was consistent with our hypothesis, that there is topographical anisotropy in the direction expected, *i.e.* parallel to the evaporating boundary line. In our previous study,[22] the evaporation was studied and we showed c-CNF alignment along (parallel) the evaporating boundary line (from edge towards the center). This indicates that suspended c-CNFs are the aligning component and are the driving component of the self-assembly. Surface examination of a film prepared from the hes-c-CNF/MWCNT dispersion (control, Fig. S4f†) shows well-dispersed MWCNTs and no aggregates were observed. The edges of the control film (Fig. 5b) and the assembled film S1 (Fig. 5a) were observed because we previously reported that the self-assembly of c-CNFs begins on the droplet boundary line.[22] SEM characterization of the film edges (Fig. 5a and b) show that, in the control film (Fig. 5b), the edges are not as uniform as in the self-assembled films (Fig. 5a). MWCNTs can be seen on the edge of the control films, while no MWCNTs are visible in the edge of the self-assembled films.
According to the Hypothesis, we expect free c-CNF alignment on the evaporating boundary line; thus, the film edge should be relatively smooth, as demonstrated in Fig. 5a. In addition, the outmost film edge is slightly thickened in the case of the self-assembled films, which is consistent with our previous observation of droplet boundary line pinning in the beginning of evaporation and accumulation of aligned fibers on the boundary edge. Therefore, a slight ‘coffee ring’ effect is expected in the very beginning of the evaporation, after which the evaporation progresses evenly as the nanosized fibers of the suspension align parallel to the boundary line. The droplet evaporates by shrinking towards the centre and allows more time for the assembly of c-CNFs in the beginning of droplet evaporation, that is, further from the droplet centre. In the previous study, we investigated the droplet evaporation of free c-CNFs in more detail, and it is known that evaporation closer to the centre is faster due to the smaller droplet volume. This likely influences the film thickness, composition, and anisotropic properties. More structural characterization is presented in ESI file Sections 1.3 and 1.4.

The dispersive effect of the c-CNFs on MWCNTs is visible in the AFM scan of the film surface (Fig. 6a), as well as of the dilute dispersion (Fig. 6b), with no visible MWCNT aggregates present. The dilute dispersion presents larger molecules, that is, MWCNTs. Hes-c-CNF in the dispersion (Fig. 6b) is smaller than c-CNFs in the supernatant (Fig. 6c) due to a higher degree of sonication. This is observed also from surface scans (ESI Fig. S5†), which confirms the presence of larger nanocellulose fibers in S1 and S2 films fabricated using supernatant c-CNFs, while S3 was fabricated using 625 kJ g\(^{-1}\) sonicated hes-c-CNF in which larger fibers are not detected. The TEM images show the c-CNF matrix rigidly attached to individual MWCNTs (Fig. 6d and e) on a torn film boundary. For comparison, Fig. 6f shows the TEM image of pristine multi-wall carbon nanotubes. Similar to SEM imaging, the challenge of TEM is to distinguish individual cellulose nanofibrils from a densely packed matrix. (S)TEM (secondary electron imaging) was used to distinguish individual c-CNFs on top of MWCNTs (Fig. 6h), from a torn film boundary (Fig. 6g). Untreated MWCNTs were examined using STEM (Fig. 6i) for comparison.

![Fig. 6 Nanostructure comparison. AFM (a–c) and (S)TEM (secondary images) (d–i) of the film surface (a), torn film boundary (d, e and g), dilute dispersion (b), dilute c-CNF supernatant (c), untreated MWCNTs (f and i) and individual MWCNTs with c-CNFs on the surface (h) from a torn nano-composite film. Scale bar sizes in (a–c) is 500 nm.](image-url)
Based on the findings of SEM and AFM examination, we can confirm that c-CNFs work as a dispersing/stabilizing agent for MWCNTs. The present study is the first to demonstrate the successful use of c-CNFs to disperse MWCNTs. However, further optimization for sonication energy per dry mass and materials' concentrations should be done.

Chemistry and interactions of the components: Interactions and chemical characterization

To attain more information regarding the effects of sonication treatments and interactions between cationic nanocellulose and carbon nanotubes, magic angle spinning (MAS) NMR spectroscopy is applied. The objectives are to examine possible chemical modification and to evaluate the interaction between c-CNFs and MWCNTs. The 1H NMR spectra in ESI Fig. S7† illustrate the hydrogen peaks of the untreated c-CNFs (Fig. S7a†) which were used as a reference for the examination of the effects of sonication treatments on the sonicated samples (Fig. S7b and c† for hes-c-CNFs, respectively, and Fig. S7d† for the hes-c-CNF-MWCNT dispersion and ESI 1.5† in more detail).

The intensity and the area of the signal peak at 2.1 ppm results from –CH2 groups of the functionalized hydroxypropyltrimethylammonium side chain and remain relatively unchanged in c-CNF samples (Fig. S7a–c†), indicating that the functional group remains intact and relatively stable throughout the sonication treatments. Untreated c-CNFs and c-CNFs sonicated with 625 kJ g⁻¹ provide the same signals. Thus, the effect of sonication on the chemical structure of c-CNF is assumed negligible. Evaluating the interaction between c-CNFs and MWCNTs during sonication is not straightforward from 1H NMR spectra (Fig. S7d†) as the analyte is merged and signals are broad and overlapping. However, when compared to the signals obtained from c-CNF alone, we can draw some conclusions on the organization of c-CNFs in the sample: the functional group is expected to point out from the entangled hes-c-CNF-MWCNT nanostructure. As the peaks in 5 ppm to 3.8 ppm are weak and broadened, the corresponding protons are not solvated, indicating strong interaction between those areas of c-CNFs with MWCNTs. The formed nanostructure is tightly packed, which prevents water from penetrating between the interaction sites. In summary, as the trimethyl protons of the cationic substituent remain unchanged in all samples, we can assume it is facing outward from MWCNTs, while the less solvated area refers to cellulose ring protons, in this case one of the H3–H5, which would be the location of the stronger interaction with MWCNTs. The result is presented in more detail in ESI 1.5.†

The NMR result is in accordance with the suggested interaction where hes-c-CNF is covering MWCNTs (Fig. 1f and 6h). The result also provides valuable information regarding the orientation of c-CNFs relative to MWCNTs, which we cannot obtain from other characterization methods. Thus, we can make assumptions regarding the dispersion chemistry that the free c-CNF encounters when suspended with the dispersion. According to NMR, there is no covalent chemical modification of the dispersion components during rather heavy sonication treatment, as also supported by the ATR-FTIR results [ESI Fig. S8†].

We propose that additional free c-CNFs are accountable for the self-assembly of c-CNF-MWCNT nanocomposite films; and this conclusion is consistent with our previous studies on the droplet evaporation of c-CNFs.22

Discussion

We demonstrated the fabrication of nanocomposite films with either isotropic or anisotropic conductivity. The Hes-c-CNF/MWCNT dispersion was used to obtain an isotropic film (control), while additional c-CNFs suspended in the dispersion drove the assembly during evaporation, resulting in an anisotropic (assembled) film. The difference between components in the control film and the assembled film is that in the latter film the dispersion and c-CNFs were suspended, while the control films were formed directly from the dispersion. Alignment observed in our previous paper22 is consistent with these results. Thus, we postulate that the free c-CNFs are responsible for the assembly along the boundary line. The suggested assembly on the evaporating liquid boundary line is described in more detail elsewhere22,32 and is consistent with the observations of Mariani et al.14 Therefore, we suggest, according to the Hypothesis, that the formation of anisotropic conductivity is due to c-CNF driven assembly.

Fig. 1 is an oversimplification of the hypothesized structure of the film in which the nanocomponents are not in scale. The diameter and length of c-CNFs have been determined using AFM and they are on average 5(±13) nm and less than 2 µm, respectively.32 According to the MWCNT manufacturer, the diameter and length of MWCNTs are on average approximately 9.5 nm and 1.5 µm, respectively. Thus, they are similar to those of untreated c-CNFs. Sonication treatment during dispersion preparation is expected to significantly fibrillate and cut the length of hes-c-CNF. AFM images of the dispersion components (Fig. 6b) and the untreated c-CNFs (Fig. 6c) are consistent with this hypothesis.

The amphiphilic nature of the nanocellulose chain affects the solubility and the surface energy, which also affects the dispersibility, the hydrophobic interactions and the aggregation tendency19,36 During sonication, applied energy affects intramolecular and intermolecular bonds, causing a different degree of fibrillation in the cellulose structure. The increasing surface area and polar/non-polar nature of cellulose aid the dispersion of hydrophobic MWCNTs in an aqueous environment. The high surface-to-length ratio of MWCNTs and the lack of functional groups determines that the chemistry of carbon nanotubes is dominated by dispersion type interactions. These extended π-conjugations, such as π–π and cation–π, enable non-covalent interactions with various substrates. Evidently, cellulose and CNTs exhibit favorable interactions toward each other so that auto aggregation of both nanoparticles does not occur, and they form a strong
entangled hybrid nanoparticle dispersion, where the cationic functional groups of c-CNFs point outwards from the hybrid structure, resulting in a stable dispersion. Findings in the SEM and NMR studies endorse that nanocellulose and carbon nanotubes have established robust connections at the molecular level and c-CNFs can be used to disperse MWCNTs. The alignment mechanism of individual c-CNFs along the evaporating droplet boundary line is based on the theory introduced by Mashkour et al., and it is based on the capillary force gradient and surface tension torque (STT) near a dry-line boundary layer. The dry-line boundary layer is the air-suspension-substrate interface, which is also referred to as a triple line.

We suggest, that in the beginning of the assembly process of the assembled films S1-S3, c-CNF fibers align along the boundary line due to pinning of one fiber end and alignment of the fiber parallel to the dry boundary line due to surface tension torques and capillary forces. Initiation of the alignment of an individual c-CNF involves surface tension forces, and the propagation of the fibre alignment, when the fibre is bent closer to the boundary line due to capillary forces, according to the alignment process described in the study of Mashkour et al. A previous study showing alignment of c-CNFs suggested that the same alignment theory applies to c-CNF during droplet evaporation. The interaction between dispersed MWCNTs and c-CNFs, as well as between the additional free c-CNFs and the hes-c-CNF-MWCNT dispersion component, is assumed to be relatively strong. The c-CNFs in the hes-c-CNF-MWCNT dispersion can further interact with free c-CNFs, which is hypothesized to initiate the assembly, further dragging the c-CNF-MWCNT into circular zones during droplet evaporation. Even though hes-c-CNF is not free to align after dispersing MWCNTs, the excess of cationic groups on the cluster surfaces allows stronger capillary forces to take place during drying when interaction with free aligning c-CNFs can take place. Therefore, free aligning c-CNFs traps along the hes-c-CNF-MWCNT entangled dispersion and an assembled structure is formed. This results in aligned c-CNFs during droplet evaporation and assembly of the hes-c-CNT-MWCNT entangled dispersion, resulting in alternating aligned c-CNFs and more entangled hes-c-CNF-MWCNTs. Sonication pre-treatment of suspended free c-CNFs increased the resistance, as indicated by the higher resistance of film S3 compared to that of film S2. This is likely due to the smaller size of the c-CNF in S3, which is a result of sonication. Thus, smaller c-CNFs seem to reduce conductive pathways more than larger c-CNFs in S2. In other words, the smaller c-CNFs of S3 may cover MWCNTs better, and therefore cause lower conductivity. It is worth highlighting, that the degree of orientation is significantly higher in the case of S3 (manufactured using hes-c-CNF + hes-c-CNF/MWCNT) compared to the control film (manufactured using hes-c-CNF/MWCNT). This confirms that the alignment is not dependent on the suspended c-CNF size, but the addition of the suspended c-CNFs. The dispersion consists of hes-c-CNF-MWCNT clusters in which caticonic groups point outwards. Therefore, the added c-CNFs can interact with these cationic groups and initiate the self-assembly at the evaporating boundary line. The added c-CNFs are expected to initiate the assembly process as described and result in alternating aligned c-CNFs and more entangled hes-c-CNF-MWCNT, shown in Fig. 1g. The anisotropic alignment is detected from the surface topography at a larger scale with image analysis, while the control films are more isotropic. Although individual aligned c-CNFs are not seen in SEM images, the detected larger scale anisotropy indicates tightly packed and aggregated aligned c-CNFs, similar to our previous study. Anisotropy of the assembled films is a result of the added c-CNFs, while MWCNTs do not show alignment in the assembled films. When evaporation has proceeded close to the end towards the centre of the film, the evaporation rate is faster due to lower volume remaining, and there is no time for assembly along the liquid boundary line in the centre part of the film.

In the present study, the purpose of the resistance measurement was to indicate the difference in the electrical properties between the control film and the self-assembled films. Furthermore, the resistance measurement was used to characterize the level of anisotropic conductivity in the assembled films. Here, two-point measurement was used since it shows the conductivity exactly between two distinct points. Even though the usage of four-point measurement rules out the possible contact resistances between the metallic probes and the CNT network, it would require homogeneous surface conductivity and, is therefore not a suitable method for characterizing anisotropic films that have circle-shaped conducting pathways.

The distinct two-probe resistance measurement points along the centre line and along the circles were repeatedly defined using a designed measurement position template (Fig. 8b). We noticed that along the circles (that is, the measurement locations in Fig. 8, measurements B, D, and E), the positive and negative probe locations could be switched without affecting the measured resistance. However, switching of the positive and negative probe locations along the centre line (that is, measurement locations in Fig. 8, measurement A and C) showed a significant difference on the measured resistance. This was further studied with IV-curves, which confirmed anisotropy along the centre line (that is, nonlinear IV-curves) in films S1–S3, while along the circular path in S1–S3 the curves were linear. Control films had linear IV-curves in both measured directions. As shown by the IR-images and resistance measurements, the conductivity of the assembled films is different along the radial direction compared to the circular zone direction. This is due to the aligned c-CNFs along the circle, while c-CNFs are packed next to each other along the neighbouring circles. Towards the centre, more and more CNFs are packed between the conducting MWCNT components, blocking the conductive pathways, and increasing resistance towards the centre. While this paper presents that the electrical properties are different along the zone and towards the centre due to c-CNF-driven assembly, the electrical properties along the radial direction should be studied further. Our observation indicates a resistive component along the
zone and a resistance coupled to a capacitive component along the radial line.

MWCNTs are often a better option for biomedical applications than SWCNTs due to more standardized methods of chemical functionalization and lower cytotoxicity.37 The c-CNF cover on the MWCNT surface could provide even better biocompatibility while still providing the benefits of MWCNTs, for example, in biomedical applications such as multifunctional antimicrobial drugs, drug delivery vehicles, functional surfaces for cell growth and new therapies for diseases.38 Furthermore, the c-CNF cover on the MWCNT surface could also provide safety for the user in terms of handling the materials. Thus, we expect that the presented cover of c-CNFs on the MWCNT surface resulting from sonochemical treatments is beneficial for the future use of MWCNTs. However, the cytocompatibility and health effects should be studied further.

Anisotropic materials are important functional materials in many fields. For instance, anisotropy in conductivity has been achieved in certain synthetic polymer systems.39,40 However, it has been more difficult to create similar materials using biologically relevant matrices.39 The size scale of c-CNFs in an anisotropic assembled substrate surface35 is optimal for diverse biomedical applications that require protein adhesion. Previously, we have shown cell adhesion and growth on c-CNF-CNT-coated cellulose mesh substrates in our study,41 in which the coating was prepared from the dispersion reported in the materials and methods section of the current paper. In addition, c-CNFs, either alone or as coating, have been shown to support cell growth, charge mediated adsorption, and cell alignment.22,42

Directionally conductive, engineered tissues have applications in a variety of fields, including stem cell biology, cardiac and neural tissue engineering, and biosensor development.39 Models to mimic native tissues, such as anisotropic myocardial fiber architecture,43 would benefit from adjustable platforms/substrates with both anisotropic and conductive properties, suggesting potential application areas for in vitro cell, tissue and disease models (organ on a chip, body on a chip, disease on a chip). In such in vitro platforms, electrical conductility could serve to stimulate cell growth, differentiation, or drastically intentional damage to the cell, in the case of disease models or photodynamic therapies.44 In addition, compartmentalized heating can be achieved with anisotropic films, as demonstrated with IR-imaging, and is adjustable by tuning film components. The demonstrated formation of assembled anisotropic conductivity is not limited to a specific substrate – that is, a circular film – nor to a specific application, but rather has potential in a variety of fields.

Experimental

Cationic cellulose nanofibers (c-CNFs) and multiwall carbon nanotubes (MWCNTs)

Cationic CNFs were produced from bleached and never-dried cellulose kraft pulp. Cationization was conducted similarly to how it was reported by Bendoraitiene et al.,44 who described the cationization of starch using EPTMAC (Raisacat, Chemigate, Finland) as a cationizing agent. The pulp was first concentrated in an oven to 63% dry matter content. The reaction mixture was prepared from 140 mL of EPTMAC, 2 g of aqueous solution of NaOH (5%), and 2.3 mL of Milli-Q water. The ingredients were mixed thoroughly and 50 mL of Milli-Q water was added to the mixture, which was warmed to 45 °C. The pulp was added to the mixture, which was then stirred for 24 h at a high cellulose consistency (~40%) with a CV Helicone Mix Flow (Design Integrated Technology Inc., Warrenton, USA) reactor. After the reaction, the cationic pulp was washed with 500 mL of ethanol, 500 mL of tetrahydrofuran (THF) (Sigma Aldrich, USA), and 1000 mL of Milli-Q water. The cationized pulp was soaked at 2% concentration of dry solids and dispersed using a high-shear Ystral X50/10 Dispermix mixer (Ystral GmH, Balrechten-Dottingen, GER) for 10 min at 2000 rpm. The pulp suspension was then fibrillated using Microfluidics microfluidizer type M110-EH (Microfluidics, Westwood, USA) at 1800 bar pressure. The suspension was processed twice through two chambers, with diameters of 400 μm and 100 μm, respectively. The fibrillated cationic CNF formed a highly viscous and transparent hydrogel with a final dry material content of 2%. The degree of substitution was analyzed according to the method proposed by Bendoraitiene et al.44 and was 0.35.

Multiwall carbon nanotubes (MWCNTs, NanoCyl 7000, Nanocyl SA., Sambreville, Belgium) were purchased from Nanocyl Inc. and the product was used in the state received. The MWCNTs are produced using catalytic chemical vapor deposition (CCVD).

Preparation of c-CNF solution, hes-c-CNF/MWCNT dispersion, and hes-c-CNF/MWCNT + free c-CNF suspensions

The cationic CNF hydrogel (2.01 w%) was diluted to 0.15 w% (W/v) concentration with Milli-Q water and sonicated for 2 min at 20% amplitude using a SONICS Vibra Cell VCX 750 ultrasonic processor (Sonics and Materials, Inc., USA). The sonicated solution was centrifuged at 10 000 g for 60 min (Thermo Scientific SL 8), and the supernatant with 0.145 w% was used for further processing. (The dry w% of the supernatant was determined from the freeze-dried product.)

The control hes-c-CNF/MWCNT samples were prepared by sonicating the c-CNF supernatant and the MWCNTs to form a homogeneous stable dispersion. The control samples contained the c-CNF supernatant with a concentration 0.05 w% of MWCNTs in aqueous medium. The total dry mass for the control sample was 0.16 g. The dispersion samples were sonicated using a tip horn sonicator Q700 (QSonica LLC, Newton, CT, USA) in 100 mL glass beakers. The amplitude of the sonication vibration was kept constant. The power output remained between 50 W and 60 W for every sonication. The sonication system included a water bath to keep samples cool during the sonication such that the temperature did not rise above 30 °C. The water bath was cooled by circulating cooling glycerol through a chiller (PerkinElmer C6 Chiller,
Samples were sonicated using a same amount of energy per dry mass, respectively 625 kJ g⁻¹. The sonication energy was chosen based on our previous studies. The resulting hes-c-CNF/MWCNT dispersion was used to prepare control nanocomposite films (Fig. 7).

The hes-c-CNF/MWCNT dispersion was further suspended (Fig. 7) with varying volume ratios of additional c-CNFs for the preparation of suspension samples S1 (37.5% v/v), S2 (20% v/v) and S3 (20% v/v). The nanocellulose used in S1 and S2 suspension is the 0.15 w% c-CNF supernatant described previously, while the nanocellulose in the S3 suspension was further sonicated with 625 kJ g⁻¹, and is thus hes-c-CNF. Thus, S1 and S2 differ in volume ratios of the dispersion and added c-CNFs, while S2 and S3 differ in pretreatment of the added c-CNFs and hes-c-CNF, respectively, while the volume ratio is constant.

Fabrication of nanocomposite films

To fabricate nanocomposite films, polydimethylsiloxane (PDMS) substrates were fabricated with a standard PDMS curing procedure in a Petri dish. A PDMS layer approximately 3 mm thick was prepared on a Petri dish surface, and then cut into 10 mm diameter circular substrates using a 10 mm punch. The nanocomposite films were prepared by casting 250 µL of the dispersion (control sample C) or suspension [samples S1, S2, S3] on PDMS substrates and drying for 5 h at 60 °C (UN55, Memmert GmbH + Co. KG, Schwabach, Germany) to obtain self-standing circular nanocomposite films with 10 mm diameter and thickness between 0.007 and 0.011 mm. The dispersion produces isotropic nanocomposite control films, while suspensions S1, S2, and S3 produce corresponding assembled anisotropic nanocomposite films. Thus, all films in this study are nanocomposite films, either isotropic or anisotropic. Later, the terms control film and assembled film are used, although when highlighting orientation, they are referred to as isotropic (control) film and anisotropic (assembled) film, respectively.

Characterization

The films were characterized using infrared imaging, resistance measurement, SEM, (S)TEM, AFM, micro-computed tomography (microCT), and nuclear magnetic resonance spectroscopy (NMR). Infrared imaging was used to illustrate the electrical anisotropy of the assembled films using heat maps, while the resistance measurement was used to demonstrate the difference between the control sample and assembled samples S1-S3 in more detail, and to investigate the electrical properties of the assembled samples. SEM, (S)TEM and AFM were used for structural characterization of the samples or their components to show or explain the assembly, as well as describe the interactions between the components. MicroCT was used for the thickness characterization of the films. Finally, NMR was performed in order to clarify the interaction between the dispersion components, as well as the effect of sonication treatments on the c-CNFs.

Infrared imaging. Infrared imaging (IR-imaging, Fluke Ti400, Everett, WA, USA) was used to obtain the electrical anisotropy of the assembled c-CNF + hes-c-CNF/MWCNT films in comparison to the uniform conductivity of the control hes-c-CNF/MWCNT nanocomposite films at the macroscale. IR-imaging was performed by applying silver ink contact to the edge of the film samples, inserting a current through the film and recording sample heating.

Electrical measurement. Resistances of nanocomposite film samples were determined according to the measurement plan shown in Fig. 8. The resistance was studied along (i) the centre line through the sample (measurements A–C) and (ii) and along imaginary circles 1 and 3 (measurements D and E, Fig. 8a), later referred to as circle 1 and circle 3, respectively. The resistance between the measurement points was deter-
mined from current outputs measured with a constant voltage 0.5 V using a using two-terminal measurement with a potentiostat (Iviumstat, Ivium Technologies B.V., Eindhoven, The Netherlands) in a chronoamperometry mode. An average of current value measured for 10 s from each measurement point was used in the resistance calculation \( R = \frac{U}{I} \). Three parallel sample films were measured, and the measurement was repeated 3–5 times for each individual sample film. Matlab was used for data analysis. To improve the repeatability of placing the measurement probes at the same locations on the parallel sample films, a mask was used. The mask (illustrated in Fig. 8b as a measurement template) was designed to include circular openings according to planned measurement locations. The openings are located along centre line in the radial direction, referred to as a radial line (locations from 1 to 9), as well as along circles 1 and 3 (locations 1, 45°, 135°, 225°, 315°, 180°, and 90°, 135°, 225°, 315°, 180°, and 90°, respectively). In the notation used in measurements A–C, \( n = p_a \) (Fig. 8) represent the locations of the negative \((n_a)\) and positive \((p_a)\) probe, respectively, in which \( a \) stands for the location of either probe on the film (Fig. 8). During measurements A and B (Fig. 8), the location of both probes varies, while in measurements C–E (Fig. 8), the negative probe remained unchanged in location 1 (measurements C and D) and in location 3 (measurement E), while the location of the positive probe was changed onto the other locations on the film. In the notation used in measurements D and E, \( n = p_a \) (Fig. 8) represent the locations of the positive probe, in which \( a \) describes the angle between the lines formed along the location of the positive probe and the centre and along the location of the negative probe and the centre, while \( \gamma \) stands for circle 1 or circle 3 and \( n \) stands for the location of the probe on the film on either one of the halves (1 for the upper half and 2 for the lower half). Locations 180°1 and 180°2 correspond to the locations 9 and 7 at the radial line, respectively. The distance of the negative probe to the positive probe in the locations 1 – 45°11 and 1 – 45°12 are equal and are thus paired in the result chart.

**Electron microscopy.** Scanning electron microscopy (SEM) and (scanning) transmission electron microscopy ((S)TEM) were used to characterize the nanomaterials used and nanocomposite films. Front and side views of the nanocomposite films were characterized using SEMs (UltraPlus, Carl Zeiss, Oberkochen, Germany; JSM-IT500, Jeol Ltd, Tokyo, Japan). The samples were attached to aluminum SEM stubs using carbon tape or carbon glue. The front view was imaged on the surface of the nanocomposite films, while the side view was characterized from the torn film edges. Torn films were placed between a string and fixed on the SEM stub to obtain a cross-section and edge views of the films. The samples were either carbon or gold-coated to avoid charging during the SEM studies. (S)TEM (JEM-F200, Jeol Ltd, Tokyo, Japan) was used to characterize the
nanomaterials used and nanocomposite films. Untreated MWCNTs and small pieces of torn nanocomposite film edges were fixed on TEM grids with a holey carbon film.

**Alignment characterization.** SEM images were collected at different locations on the film to perform SEM image-based orientation analysis. Cytospectre software\(^4^5\) was used for the analysis. A parameter called circular variance (CV) was determined from each image in order to describe to degree of orientation. A CV value of 1 refers to perfect isotropy, while a value of 0 is perfect anisotropy. In addition to the CV value, a mean orientation angle of each image location was determined in order to show the orientation direction along the evaporating boundary line. While CV only describes the degree of orientation – that is, isotropy or anisotropy – the mean orientation can be used to show if the orientation direction is according to our hypothesis; that is, along the evaporation boundary line, and thus along the imaginary circles.

**MicroCT.** Nanocomposite films were imaged with X-ray microtomography (microCT) using MicroXCT-400 (X-ray tube voltage of 40 kV and a current of 250 µA; Carl Zeiss X-ray Microscopy, Inc., Pleasanton, CA, USA). The imaged sample area was 2.98 mm\(^2\). 3D microCT images were reconstructed from 1601 projections with a 10 s exposure time (20× objective, binning 2, pixel size 1.048 µm) using the microCT software tool XMReconstructor. 3D image stacks were manually thresholded for the 3D analysis. The mean film thickness with standard deviations from different locations of the films was calculated with the BoneJ Fiji plugin.\(^4^5\) The data visualization was realized using Avizo 2019.3 software (Thermo Fisher Scientific, Waltham, MA, USA).

**Atomic force microscopy.** Atomic force microscopy scanning was performed in a tapping mode using a standard ACTA AFM probe with a resolution of 256 × 256 pixels. Images were analyzed and postprocessed using AFM image processing software XEI (Park Systems, USA).

**Nuclear magnetic resonance spectroscopy (NMR).** The following samples were prepared and freeze-dried for NMR studies. The cationic CNF hydrogel (2.01 w%) was diluted to 0.15 w% (W/V) concentration in D\(_2\)O (Sigma Aldrich), sample 1 (untreated c-CNF). Samples 2–4 were sonicated for 2 min at 20% amplitude using a SONICS Vibra Cell VCX 750 ultrasonic processor (Sonics and Materials, Inc., USA), with subsequent centrifugation at 10 000 g for 60 min (Thermo Scientific SL 8). The resulting supernatant was sonicated using 625 kJ g\(^{-1}\) or 1250 kJ g\(^{-1}\) and a tip horn sonicator Q700 (QSonica LLC, Newton, CT, USA), resulting in samples 2 and 3 (c-CNF supernatant sonicated 625 kJ g\(^{-1}\) or 1250 kJ g\(^{-1}\), respectively). Sample 4 (sonicated c-CNF-MWCNT dispersion) was prepared by sonicating the c-CNF supernatant (in D\(_2\)O) and MWCNTs using 625 kJ g\(^{-1}\). Samples 1–4 were freeze-dried to remove excess moisture and used in NMR experiments.

NMR spectra were measured on a 500 MHz JEOl JNM-ECZ 500R spectrometer. Samples (approx. 50 mg) were packed into 3.2 mm diameter zirconia rotors with KelF caps as a tick suspension in D\(_2\)O. The semi-solid state FG-MAS 1H spectra were recorded at room temperature, with the high-resolution field gradient FG-MAS probe at a spinning rate of 5 kHz. A water suppression pulse sequence was applied during the measurements.

**Conclusions**

Application of sonochemical treatments to aqueous mixtures of cationic nanofibrillated cellulose and carbon nanotubes leads to even and relatively stable solutions of the assembled components, which can be easily formed as free-standing conductive nanocomposite films. Diverse characterization techniques confirm the strong interactions between c-CNFs and MWCNTs, mainly occurring through ionic and hydrophobic interactions, as well as hydrogen bonding induced by sonochemical treatments. Sonochemical treatment induces robust interactions between c-CNFs and MWCNTs in aqueous media, forming a strong entangled hybrid nanoparticle dispersion hes-c-CNF/MWCNT, where cationic functional groups of hes-CNFs point outwards from the hybrid structure. Drying of the dispersion results in evenly conducting films, while the incorporation of free c-CNFs (S1, S2) or hes-c-CNFs (S3) makes it possible to control the self-assembly of nanomaterials along the evaporating dry-line boundary and the simultaneous formation of conducting zones and thus the directional increase in resistance in the radial direction. The amount and composition of the added c-CNFs (or hes-c-CNFs) influence the conductivity differences in different directions. These films can be considered as free-standing self-assembled nanocomposite films displaying a tunable surface with functional properties such as adjustable conductivity. The novelty in the method described herein is that it produces adjustable directional conductivity and uses c-CNFs as a dispersing agent. The method is not limited to a specific application or to nanocomposite films as a substrate. Instead, the tunable composition, substrate preparation technique, and functionalization possibilities offer interesting possibilities in the fields of biomedical sciences and energy applications in general and should be studied further.

**Author contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Conflicts of interest**

There are no conflicts of interest to declare.

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