Engineering Nanocellulose Biointerfaces Toward Bioactivity and Strength

Maija Vuoriluoto
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Maija Vuoriluoto

A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Chemical Engineering, at a public examination held at the lecture hall Ke2 of the school on 29th of September 2017 at 12.

Aalto University
School of Chemical Engineering
Department of Bioproducts and Biosystems
Bio-based Colloids and Materials
Supervising professor
Prof. Orlando J. Rojas, Aalto University, Finland

Thesis advisor
Dr. Hannes Orelma, VTT Technical Research Centre of Finland, Finland

Preliminary examiners
Dr. J. Vincent Edwards, United States Department of Agriculture, USA
Prof. Harry Brumer, The University of British Columbia, Canada

Opponent
Prof. Robert D. Tilton, Carnegie Mellon University, USA
Abstract

This work was focused on developing bioactive materials from cellulose, mainly in the form of nanofibrils (CNF). The main efforts involved modifications to adjust the surface behavior (adsorption and fouling) and wet strength of CNF in various structures. In addition to other surface analytical techniques, ultrathin films of CNF were investigated by surface plasmon resonance (SPR) and quartz crystal microbalance with dissipation monitoring (QCM-D). Additionally, CNF nanopapers and wet-spun filaments were prepared and modified with functional properties, including bioactivity.

The effect of molecular architecture of block and random copolymers of poly(2-(dimethylamino)ethyl methacrylate) (PDMEAMA) and poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) on adsorption on substrates with low, medium and high density of electrostatic charges, namely, regenerated cellulose, CNF and TEMPO-oxidized CNF (TOCNF) was investigated. Polymer adsorption was mainly driven by electrostatic interactions between anionic groups of the cellulosic materials and cationic segments of the copolymers. Charge neutralization upon adsorption of block copolymers onto TOCNF was accompanied with significant water expulsion from the interface. The copolymers were highly efficient in producing antifouling TOCNF surfaces by reducing non-specific human IgG adsorption. The blocking efficiency was determined to be between 84-100%, depending on the polymer architecture. Remarkably, the copolymer passivation did not impair the selectivity and sensitivity of the TOCNF biointerfaces toward anti-human IgG after complementary bioactive molecules were installed by EDC/NHS coupling. The copolymer passivation reduced the otherwise nine-fold false response by the biointerface. Water-resistant CNF was prepared by TEMPO-oxidation and EDC/NHS coupling of aminobenzophenone to CNF (BP-CNF). Nanopapers and wet-spun filaments with superior wet strength (230-fold increase) were prepared from BP-CNF upon UV-activated crosslinking. The BP-CNF material was suitable for a secondary activation cycle with EDC/NHS to introduce bioactivity without significant interference from the BP functionalization. An anti-hemoglobin biointerface prepared on BP-CNF presented excellent affinity with hemoglobin, yet minimal non-specific adsorption as probed with human serum albumin. The results point to the possibility of tuning the systems’ sensitivity and selectivity by passivation with random copolymers. Additionally, the BP-CNF filaments exhibiting anti-hemoglobin biointerfaces were employed successfully in testing of hemoglobin with fluorescence-labelled secondary antibodies. Overall, the work presented a method to adjust the material properties of cellulosic nanomaterials to allow their adoption in biomedical applications and biosensor development, without compromising the potential of the material for bioactivation.

Keywords cellulose nanofibrils, copolymer adsorption, bioactivity, biointerfaces, antifouling, UV-activated crosslinking, wet strength, nanopapers, filaments
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Artikkeli
Essee

Tiivistelmä
Tässä työssä tutkittiin bioaktiivisten materiaalien kehittämistä selluloosananofibrilleistä. Työn tarkoituksena oli muokata nanofibraarisen selluloosamateriaalin pintoaminaisuuksia, kuten proteiinin adsorptiota ja hylkivyyttä siihen, sekä sen määrälukuuta erilaisissa rakenteissa. Työssä hyödynnettiin pääasiassa nanofibrillarista selluloosasta valmistettuja ohutkalvoja, joita analysoitiin muun muassa pintaherkkien pintaplasmoresonanssin- (SPR) ja kvartsiiden mikrovaakamenetelmien (QCM-D) avulla. Myös nanopaperia ja märkäkehrityjä selluloosafilaamentteja tavoitellujen ominaisuuksien valmistettiin ja tutkittiin.


Avainsanat
selluloosananofibrillit, kopolymeeriadsorptio, bioaktiivisuus, biorajapinnat, UV-aktiivoin ristisotominen, määrälukuus, nanopaperit, filamentit

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Preface

This doctoral dissertation was carried out in the Bio-based Colloids and Materials research group at the Department of Bioproducts and Biosystems (formerly Department of Forest Products Technology) of the School of Chemical Engineering in Aalto University between years 2013 and 2017. Funding by the Academy of Finland through its Centres of Excellence Programme (2014-2019) “HYBER” and also under Project “Microfluidics” is gratefully acknowledged. Support by TES – Tekniikan edistämissäätiö (Finnish Foundation for Technology Promotion) is also highly appreciated.

I want to express my deepest gratitude to my supervisor Prof. Orlando J. Rojas for giving me the opportunity to work in his group and for his continued support and invaluable advice and guidance throughout this process. He is always available for his students and his endless positive attitude is an inspiration to us all. I would also like to thank Prof. Janne Laine for the initial chance to start my doctoral studies in his group.

My most heartfelt gratitude goes to my thesis advisor Dr. Hannes Orelma. Without his invaluable input and continuous support and belief in me as well as patience for my never-ending questions, this dissertation would never have been possible. Not only has he taught me about science and research but also about life.

I would like thank all my co-authors for their insightful input and expertise in refining my research. For the laboratory technicians Ritva Kivelä, Marja Kärkkäinen, Rita Hatakka and Anu Anttila I would like to express my sincerest gratitude for running the labs and for the excellent ever available assistance. All colleagues at the department are thanked for creating such a pleasant atmosphere and workplace. I want to thank all past and present BiCMat members for the warm and supportive work environment in our group.

Additionally, special thanks go to the coffee break and lunch groups, Elli, Reeta, Emmi, Jonna, Saija, Juuso, Laura, Katri, Karoliina, Iina and Ansku. Thank you for the sometimes scientific but more often very unscientific discussions and memorable moments both at the department and elsewhere.

I would also like to thank my family and friends for providing much-needed balance from work, I would not have managed this without your love and support. Especially, Äiti, Iskä, and Mikko, thank you for always believing in me and giving me the confidence to follow my dreams.
Finally, I want to thank my daughter Vilma for the immense joy, happiness and occasional mayhem she has brought my life. You have taught me more about myself than I ever thought possible. I am so proud to be your mother!

Words cannot express how grateful I am to my husband Tuomas for all the unwavering love, patience and encouragement throughout the years. Without you, I would be lost. Thank you for taking care of our family, you are my everything!

Espoo, August 28th, 2017
Maija Vuoriluoto
List of Publications

This doctoral dissertation consists of a summary and of the following publications, which are referred to in the text by their Roman numerals.

**Paper I.** Vuoriluoto, Maija; Orelma, Hannes; Johansson, Leena-Sisko; Zhu, Baolei; Poutanen, Mikko; Walther, Andreas; Laine, Janne; Rojas, Orlando J. (2015) Effect of Molecular Architecture of PDMAEMA–POEGMA Random and Block Copolymers on Their Adsorption on Regenerated and Anionic Nanocelluloses and Evidence of Interfacial Water Expulsion. *Journal of Physical Chemistry B, 119*(49), 15275–15286, 10.1021/acs.jpcb.5b07628

**Paper II.** Vuoriluoto, Maija; Orelma, Hannes; Zhu, Baolei; Johansson, Leena-Sisko; Rojas, Orlando J. (2016) Control of Protein Affinity of Bioactive Nanocellulose and Passivation using Engineered Block and Random Copolymers. *ACS Applied Materials and Interfaces, 8*(8), 5668–5678, 10.1021/acsami.5b11737

**Paper III.** Orelma, Hannes; Vuoriluoto, Maija; Johansson, Leena-Sisko; Campbell, Joseph; Filpponen, Ilari; Biesalski, Markus; Rojas, Orlando J. (2016) Preparation of Photoreactive Nanocellulosic Materials via Benzophenone Grafting. *RSC Advances, 6*, 85100-85106, 10.1039/C6RA15015B

**Paper IV.** Vuoriluoto, Maija; Orelma, Hannes; Lundahl, Meri; Borghei, Maryam; Rojas, Orlando J. (2017) Filaments with Affinity Binding and Wet Strength Can Be Achieved by Spinning Bifunctional Cellulose Nanofibrils. *Biomacromolecules, 18*(6), 1803–1813, 10.1021/acs.biomac.7b00256
Author’s Contribution

**Paper I**: Effect of Molecular Architecture of PDMAEMA–POEGMA Random and Block Copolymers on Their Adsorption on Regenerated and Anionic Nanocelluloses and Evidence of Interfacial Water Expulsion

Maija Vuoriluoto was responsible for the experimental design, performed the main part of the experimental work, including cellulose ultrathin film preparation, QCM-D, SPR, and AFM. She analyzed the results and wrote the manuscript as principal author under supervision of Dr. Hannes Orelma and Prof. Orlando Rojas. Dr. Baolei Zhu was responsible for polymerization and characterization of block and random copolymers and Dr. Leena-Sisko Johansson for XPS measurements. M.Sc.(Tech.) Mikko Poutanen and Prof. Andreas Walther collaborated with the interpretation of results and writing the manuscript.

**Paper II**: Control of Protein Affinity of Bioactive Nanocellulose and Passivation using Engineered Block and Random Copolymers

Maija Vuoriluoto was responsible for the experimental design, performed the experimental work, analyzed the results, and wrote the manuscript as principal author under supervision of Dr. Hannes Orelma and Prof. Orlando Rojas. Dr. Baolei Zhu was responsible for polymerization and Dr. Leena-Sisko Johansson for XPS measurements.

**Paper III**: Preparation of Photoreactive Nanocellulosic Materials via Benzo-phenone Grafting.

Maija Vuoriluoto was responsible for experimental design together with co-authors, performed the tensile testing and AFM imaging, analyzed the corresponding results, and wrote the manuscript together with the co-authors.

**Paper IV**: Filaments with Affinity Binding and Wet strength can be achieved by Spinning Bifunctional Cellulose Nanofibrils

Maija Vuoriluoto was responsible for the experimental design together with Dr. Hannes Orelma and performed the main part of experimental work, including tensile testing, cellulose ultrathin film preparation, SPR, QCM-D, AFM, and CLSM. She analyzed the results and wrote the manuscript as principal author under supervision of Dr. Hannes Orelma and Prof. Orlando Rojas. Dr. Hannes Orelma was responsible for part of QCM-D measurements (<10%), Dr. Maryam Borghei for SEM imaging, and M.Sc.(Tech.) Meri Lundahl for wet-spinning.
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AGU</td>
<td>β-D-anhydroglucopyranose</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>BP</td>
<td>benzophenone</td>
</tr>
<tr>
<td>BP-CNF</td>
<td>benzophenone-functionalized TEMPO-oxidized cellulose nanofibrils</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CNF</td>
<td>cellulose nanofibrils</td>
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<tr>
<td>DMAEMA</td>
<td>2-(dimethylamino)ethyl methacrylate</td>
</tr>
<tr>
<td>DS</td>
<td>degree of substitution</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>hIgG</td>
<td>human immunoglobulin G</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>OEGMA</td>
<td>oligo(ethylene glycol)methyl ether methacrylate</td>
</tr>
<tr>
<td>PEG-amine</td>
<td>methoxypolyethylene glycol amine</td>
</tr>
<tr>
<td>RMS</td>
<td>root mean square</td>
</tr>
<tr>
<td>TEMPO</td>
<td>(2,2,6,6-tetramethyl-piperidin-1-yl)oxyl</td>
</tr>
<tr>
<td>TOCNF</td>
<td>TEMPO-oxidized cellulose nanofibrils</td>
</tr>
<tr>
<td>TMSC</td>
<td>trimethylsilyl cellulose</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>QCM-D</td>
<td>quartz crystal microbalance with dissipation monitoring</td>
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<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
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“A learning experience is one of those things that says, ‘You know that thing you just did? Don’t do that.’”

— Douglas Adams, The Salmon of Doubt
1. Introduction

Forest products have always played a significant role in the Finnish economy, which nowadays expands to new developments, for example, toward advanced materials from such renewable and sustainable resources. Especially in the biomedical field, new innovations require generation of efficient material platforms with biocompatibility and added functionalities. Cellulosic nanomaterials exhibit significant potential to develop these types of materials as they display a large surface area, low toxicity, thermo-mechanical resistance, and hydrophilicity. Isolation of nanocellulosic materials from wood was first reported already in the 1980s (Turbak et al. 1983) but large-scale production has not been attempted until recently. At present, production from laboratory to pilot or semi-industrial scales has been taken and, although many obstacles need to be overcome, there is promise for a wide utilization of nanocellulose in the future.

Recently, the interest in utilizing traditional cellulose-based material, in the form of paper or cotton threads, in developing novel biosensors for point-of-care diagnostics has increased exponentially (Yetisen et al. 2013, Nilghaz et al. 2013). In addition, nanocellulosic materials have been proposed to offer unique possibilities in tissue engineering, implantable diagnostics, and drug delivery (Rojas et al. 2015). Thus, the objective of this work was to develop new multifunctional nanofibrillar cellulose platforms for potential utilization in novel biomedical applications. The aim was to study surface interactions of cellulose nanofibril materials and to tailor them toward specific features, such as low fouling, wet mechanical strength, and bioactivity. Due to the stability and relative inertness of cellulose in aqueous media, surface modification was required for functionalization, including robust attachment of biomolecules for bioactivity. Such functionalization strategies, however, are often detrimental to other desired properties of the material, e.g., wet strength. Highly reactive surfaces also typically exhibit significant non-specific protein adsorption or fouling, which is problematic especially when designing bioassays or implantable devices.

As a general overview of the work conducted in this thesis, Papers I and III mainly focus on investigating and adjusting the properties of the cellulosic materials for feasible utilization in biomedical applications. Papers II and IV investigate how the modified properties affect bioactivity (Figure 1).
**Paper I** considers the effect of the detailed molecular architecture of block and random copolymers comprising hydrophilic oligo(ethylene glycol) methyl ether methacrylate (OEGMA) and positively charged 2-(dimethylamino)ethyl methacrylate (DMAEMA) monomers on adsorption onto cellulosic materials. It was shown that the cationic segments of the copolymers functioned as anchors for the hydrophilic segments onto highly-charged nanocellulose. Interestingly, the extending hydrophilic segments endowed the cellulose surface with anti-fouling properties against proteins. The work in **Paper II** includes a discussion about the effect of copolymer modification to produce protein-repellent cellulose surfaces. The copolymers were utilized for total human immunoglobulin G rejection on highly charged TEMPO-oxidized cellulose nanofibrils (TOCNF), while maintaining good levels of specific antibody-antigen interactions with prepared biointerfaces.

Biointerfaces developed on nanocellulose requires functionalization by, e.g., TEMPO-oxidation but this often implies a reduction in wet mechanical properties of the material. The focus of **Paper III** was to improve the wet strength of cellulose nanofibril materials while maintaining sufficient amount of reactive sites on the surface for further bioactivation. It was found that conjugation of photoreactive benzophenone groups onto TOCNF dramatically increased the wet mechanical strength of nanopapers upon UV-activated photocrosslinking. Wet-spinning of the developed functionalized nanocellulose was demonstrated in **Paper IV**. It was determined that benzophenone-activation did not significantly impair the potential of the material for further activation and the bifunctional filaments displayed simultaneously wet strength and bioactivity.
2. Background

2.1 Cellulose in biomedical applications

Development of high-performance, advanced materials from renewable resources has become paramount in decreasing the use of non-renewable products. Especially, innovations in areas such as biofiltration and affinity separation (Orelma et al. 2014), drug delivery (Li et al. 2012) and cell therapy (Mertanen et al. 2016) are emerging in the biomedical and environmental fields, all of which demand the development of new functional materials. Additionally, advanced, high-efficiency materials are needed in tissue engineering (Tamayol et al. 2013), microbiological applications, wound dressing, and in designing biosensors and diagnostic assays (Aikio et al. 2006, Pelton 2009) as well as implants (Mostafalu et al. 2016). Recently, native cellulose fibers and nanocellulose products have received significant attention as sustainable substrates for these types of applications.

2.1.1 Diagnostic assays

Public health issues and the urge to improve quality of life have led to an increased demand for rapid, inexpensive, easy-to-use point-of-care (POC) assays. The established diagnostic assay formats at present include dipsticks and lateral flow assays (LFA). Dipsticks, such as the pH strip, rely on blotting of the sample onto an activated substrate with pre-stored reagents. Dipsticks provide semiquantitative results with colorimetric charts or detection equipment but lack the sophistication to design more complex assays (Parolo, Merkoçi 2013). LFAs, on the other hand, are based on lateral flow of liquid through a porous membrane. The liquid sample passes through various zones where molecules with specific interactions with the analyte have been attached. If antibodies are utilized exclusively as recognition elements, the tests are called lateral flow immunoassays (LFIA). The most common example of a LFA is the home pregnancy test (Fridley et al. 2013). Commercial LFAs have also expanded from traditional diagnostics to food safety, environmental monitoring, veterinary medicine, biodefence, and drug testing (Yetisen et al. 2013).

The first immunodiagnostic tests with radioactively labelled proteins were reported already in the late 1950s (Yalow, Berson 1959) but due to obvious health risks associated with this method, an enzyme-linked immunosorbent assay (ELISA) was introduced in 1971 (Engvall, Perlmann). ELISA is still one of the
most important methods based on a sandwich-type of detection and is used for screening several human diseases in laboratories (Lequin 2005) (Figure 2).

![Schematic illustration of enzyme linked immunosorbent assay (ELISA) for detection and reporting of antigens.](image)

In the ELISA test, first an antibody or an antibody fragment is immobilized onto a support surface. The immobilized primary antibody captures an antigen upon exposure to the test solution while excess is washed away. A secondary antibody conjugated with an enzyme is then introduced on the test surface and is expected to bind to the captured antigen. The conjugated enzyme functions as a catalyst when the surface is treated with a substrate solution and a fluorescent or colored product is formed. Quantitative information can be obtained by ELISA if incorporated with instrumentation to carefully assess color or fluorescence intensity. Due to multiple steps and the need for expensive laboratory equipment, ELISA is mainly utilized in laboratory environments. However, many LFAs rely on similar principles as those in the ELISA method for detection but reporting of the capture event is provided by other methods, e.g., gold nanoparticles, radioactivity, or magnetic sensing (Yager et al. 2006).

The typical structure of an LFIA is presented in Figure 3. Briefly, the liquid sample with the target analyte is introduced to a sample pad (Posthuma-Trumpie et al. 2009). The sample wicks to a conjugate release pad where, depending on the application, the labelled analyte or recognition elements are dried, which interact specifically with the target analyte, if present in the sample. The sample flows by capillary force to the reaction membrane or detection pad, where test and control lines have been sprayed. The target analyte is detected at the test line and results in a response, such as color change. The control line confirms the proper functioning of the test strip. Excess sample is wicked by the adsorbent pad at the end of the strip.
The reaction membranes of LFAs are typically made from nitrocellulose. Nitrocellulose is manufactured by treating cellulose with, e.g., nitric acid and the hydroxyl groups of cellulose are esterified with nitrate groups. Porous membranes can be casted from nitrocellulose that facilitate the spontaneous wicking of fluids (Tonkinson, Stillman 2002, Posthuma-Trumpie et al. 2009, Fridley et al. 2013). Utilization of nitrocellulose membranes as substrates for biomolecule immobilization began in the 1960s when Nygaard and Hall (1963) discovered that free RNA strands would pass through a nitrocellulose filter as opposed to RNA-DNA duplexes, which would adsorb to the filter. A breakthrough came when Southern (1975) demonstrated the immobilization of DNA on nitrocellulose from agarose gel by capillary transfer. This method, today called “Southern blotting”, enabled the immobilization and analysis of nucleic acid segments of specific length.

Nitrocellulose-based LFAs commonly give results rapidly, in less than 15 minutes. Furthermore, they are relatively simple to manufacture and do not require refrigeration. However, nitrocellulose membranes are typically fragile and require the support of a plastic layer for cutting and handling. In addition, the nitrocellulose-based LFA strips are commonly encased in plastic holders for better robustness (Posthuma-Trumpie et al. 2009). Furthermore, the exact mechanism of biomolecule affinity with nitrocellulose is still under debate but, regardless of the mechanism, it is well known that biomolecules adsorb onto the nitrocellulose membranes (Fridley et al. 2013, Tonkinson, Stillman 2002). The significant non-specific adsorption of proteins demands blocking agents to be applied on the membrane and thus reduce the risk of a false response. Importantly, some of the limitations of nitrocellulose-based LFAs include lower sensitivity compared to cultures, lack of multiplexability, and the fact that qualitative results require special manufacturing techniques (Fridley et al. 2013). To
overcome the need for external plastic supports and to address the inadequate sensitivity and specificity of nitrocellulose-based LFAs, interest in developing new sophisticated devices with additional functionalities has emerged (Posthuma-Trumpie et al. 2009, Cazacu et al. 2003). These include, among others, two-dimensional “paper” (nitrocellulose) networks (2DPNs) that feature automated multistep processes (Fu et al. 2010, Fu et al. 2011b, Fu et al. 2011a, Fu et al. 2012) as well as paper-based (Yetisen et al. 2013, Cate et al. 2015) and thread-based (Nilghaz et al. 2013) microfluidic devices.

2.1.2 Novel biomedical applications

In addition to traditional diagnostic sensors, novel biomedical applications are emerging, creating a need for engineered advanced and well-defined functional materials from renewable resources. Among these emerging biomedical areas, one can cite stem cell therapy and drug delivery, tissue engineering as well as implants, and implantable biosensors. Recent efforts in implantable drug delivery have introduced novel sutures that can be used to administer to target tissues in a controlled manner, antibiotic substances (Li et al. 2012), proteins (Fuchs et al. 2012) or antibodies (Shibuya et al. 1999). Also, the delivery of stem cells to the desired tissue to enhance tissue regeneration and reduce inflammation has been demonstrated with surgical sutures (Guyette et al. 2013, Reckhenrich et al. 2014) and scaffolds (Voss et al. 2016).

Implantable diagnostic devices or biosensors are expected to function as devices that produce continuous information about analyte concentration or conditions to significantly widen the potential for treating and diagnosing diseases and reduce the need for invasive procedures, such as blood sampling and biopsies (Vaddiraju et al. 2010, Micheli et al. 2013). Tissue engineering is also a field with a need for biocompatible functional materials. Development of, e.g., organ substitutes based on extracellular matrices grown on spun filament networks comprise an interesting and challenging novel material platform (Murugan, Ramakrishna 2006). In the environmental engineering area, bioaffinity separation has proved to be beneficial in reducing the accumulation of pharmaceutical residues, heavy metal and endocrine disrupting compounds in waste water systems (Miège et al. 2008, Benotti et al. 2008). Overall, there is a growing need for highly-engineered functional, biodegradable materials for various applications from sustainable and renewable resources.

2.1.3 Fiber-based materials in biomedical applications

Cellulose fiber-based materials present a sustainable and biodegradable alternative for biomedical applications due to their many advantageous properties, such as hydrophilicity, low toxicity, recyclability, and availability. Cellulose fiber-based films and threads also facilitate the utilization of lateral flow as the porous structure enables spontaneous wicking of liquids due to capillary forces. The first diagnostic test based on paper for determination of glucose in urine was presented already in 1957 by Free at al. (Free et al. 1957) but lateral flow was incorporated into paper-based tests only in 1985 (Zuk et al.). More recently,
concepts of microfluidic paper-based analytical devices (μPADs) and bioactive paper have been introduced (Martínez et al. 2007, Su et al. 2007). The μPADs allowed easier multi-analyte determination with fabrication of hydrophobic channels for simultaneous, multiple analyte detection. Since then, the μPADs have evolved into complex analytical devices incorporating 3D structures and multi-step processes that can be operated without external equipment. Research surrounding paper-based biosensors has increased substantially in recent years. This subject is discussed in several comprehensive reviews, such as those by Pelton (2009), Yetisen et al. (2013), Cate et al. (2015) and Yang et al. (2017).

Thread-based microfluidic devices incorporate similar principles as the μPADs but in a thread format, as multifilament cotton threads also wick fluids spontaneously (Li et al. 2010, Reches et al. 2010). They offer opportunities in multiplexation without need for hydrophobic channeling and allow for minimal sample volumes. Such systems also display better tensile strength and flexibility compared to 2D paper-based devices and make 3D structures possible (Nilghaz et al. 2013). As the interest in cellulose fiber-based diagnostic devices grows, it is also important to gain better understanding about the interfacial interactions in such systems and to tune the cellulosic substrate properties to desired directions to allow for sophisticated, more effective application development.

Innovations in biomedical fields, such as diagnostics, drug delivery, tissue engineering, and cell therapy demand biocompatible, high-efficiency advanced functional materials for emerging applications, as already discussed. Cellulose fiber-based products, and especially nanocellulose, are ideally suited for development of these kinds of materials due to their unique properties, such as thermo-mechanical resistance, low cytotoxicity, biocompatibility, and large surface area. Potential application fields for nanocellulose-based bioactive systems include drug delivery, biosensors and diagnostics, medical implants, antimicrobial membranes, skin tissue repair, vascular grafts and scaffolds (Figure 4).
Cellulose nanofibrils (CNF) (Arola et al. 2012), cellulose nanocrystals (CNC) (Habibi et al. 2010, Edwards et al. 2013, Zhou et al. 2015) and bacterial cellulose (BC) (Klemm et al. 2001) have been utilized for the development of bioactive systems in, e.g., diagnostics and biosensors (Orelma et al. 2012a) and biofiltration membranes (Orelma et al. 2014, Sirviö et al. 2016). Moreover, recent efforts in this area have led to implantable diagnostic devices to monitor local tissue environments, such as cotton thread-based sutures for pH, glucose, or stress sensing (Mostafalu et al. 2016). Another area with significant interest in bioactive interfaces is wound dressing for monitoring local tissue environment. Nanocellulose-based materials and aerogels have been demonstrated for enzyme detection and sequestration with fluorescence reporting (Derikvand et al. 2016, Edwards et al. 2016). Furthermore, surgical sutures produced by wet-spinning of CNF filaments have been demonstrated to function as stem cell carriers (Mertaniemi et al. 2016). Additionally, the spinning of native cellulose filaments offers opportunities in tissue engineering to grow extracellular matrices for the development of organ substitutes (Tamayol et al. 2013). Plant-derived nanocellulose hydrogels have also been demonstrated for 3D cell cultures in tissue engineering that allow the study and development of organ transplants in 3D mimicking the native extracellular matrix instead of 2D formats (Bhattacharya et al. 2012, Liu et al. 2016). Further information on bioactive nanocellulose is provided by Zhang et al. (2013c) and Rojas et al. (2015).
2.2 Lignocellulose and cellulose nanofibrils

The function of the cellulose biopolymer is mainly to provide mechanical strength to wood, annual plants, some marine creatures, algae, and bacteria (Gandini 2011, Moon et al. 2011). Even though cellulose was first reported in 1833 (Payen, Persoz), its structure and chemistry is still under debate despite significant scientific interest.

2.2.1 Cellulose chemistry

Cellulose is a linear homopolymer consisting of \(\alpha\)-anhydroglucopyranose units (AGU) linked together by glycosidic \(\beta-(1\rightarrow4)\) bonds in a \(\text{C}_4\) chair conformation (Klemm et al. 1998, Gandini 2011). An illustration of the structure of a cellulose chain is presented in Figure 5.

Cellulose is a syndiotactic polymer when the basic unit is considered to be the anhydroglucose unit. However, the repeating unit of cellulose is sometimes proposed to be the dimer called cellbiose composed of two AGU monomers, composing an isotactic polymer. The hydroxyl groups on the AGUs are located at the C2, C3, and C6 positions (Figure 5) (Klemm et al. 1998). The length i.e. degree of polymerization (DP) of cellulose is defined by the number of AGUs linked together (Kontturi et al. 2006). Cellulose is a polydisperse material and the DP varies significantly from hundreds to thousands, depending on the source species and on the isolation and purification procedures (Gandini 2011, Klemm et al. 1998). Cellulose also differs greatly from its monomer water-soluble glucose. Already at DP 6 it is insoluble in water due to strong tendency to form intra- and intermolecular hydrogen bonds (Kontturi et al. 2006, Klemm et al. 1998).

The supramolecular chemistry of cellulose is very complex. There are four known crystalline polymorphs of cellulose, namely, I, II, III and IV (Moon et al. 2011, Nishiyama et al. 2002). Cellulose I is the “natural” or “native” cellulose form that is produced in a variety of organisms, e.g., in trees, plants, bacteria and algae (Moon et al. 2011). Hydrogen bonds exist between O3–H and O5 and between O2–H and O6 (Figure 5) in cellulose I (Klemm et al. 1998, Nishiyama et al. 2002, Nishiyama et al. 2003). These hydrogen bonds are responsible for stabilizing the two-fold helix conformation of crystalline cellulose and for the substantial stiffness of the cellulose chain. Cellulose I has been found to exhibit two allomorphs I\(\alpha\) and I\(\beta\) (Atalla, VanderHart 1984). The dominating crystalline structure for bacterial and most algae cellulose is I\(\alpha\) and for higher plant wall
cellulose e.g. for ramie, cotton, wood and tunicates the dominating structure is $I_\beta$ (Moon et al. 2011, Klemm et al. 1998). Cellulose $I_\alpha$ phase is described as a triclinic $P1$ structure with one cellulose chain in a unit cell whereas the cellulose $I_\beta$ phase has a monoclinic $P2_1$ structure and two conformationally distinct chains in a unit cell. These unit cells imply that the basic structural difference of these allomorphs is caused by how the consecutive hydrogen-bonded planes are organized on top of each other (Nishiyama 2009).

In cellulose biosynthesis in plants and trees, the cell walls’ cellulose synthesis complexes with 6 subunits each, extrude 3 cellulose chains held together by van der Waals forces (Vandavasi et al. 2016). These cellulose chains then assemble into elementary fibrils that consists of 18 cellulose chains where planar cellulose chain sheets are bound together by intermolecular hydrogen bonds that are connected via interplanar van der Waals forces (Moon et al. 2011, Sjöström 1993, Nishiyama et al. 2002, Maleki et al. 2016). The cross-section of the elementary fibril is rectangular with dimensions varying between 3-5 nm. The elementary fibrils further consolidate to form microfibrils with a length that can reach micrometers (Moon et al. 2011, Klemm et al. 1998). Figure 6 presents a top-down image of the hierarchical structure of wood.

The macromolecular structure of cellulose is not uniform throughout the fiber (Klemm et al. 1998). At present, a two-phase model is used to describe the cellulose structure consisting of low ordered or amorphous and high ordered or crystalline regions. An illustration of the possible configuration of these regions is seen in Figure 6. The crystallinity of cellulose is important when considering its solubility in water. Because of the abundance of hydroxyl groups and their
interaction with water molecules, cellulose is hydrophilic, but it does not dissolve in water (Klemm et al. 1998, Kontturi et al. 2006, Medronho et al. 2012). However, water is able to penetrate and disrupt the intermolecular hydrogen bonding of the amorphous regions and cause swelling although it cannot penetrate into the crystalline regions. The fiber wall cellulose has a high degree of crystallinity, about 60 - 75% (Alén 2000).

Macrofibrils are the aggregates of microfibrils, which have diameters in the micrometer scale and are the construction units for the fiber cell wall architecture (Figure 6). The cell wall of a wood fiber basically consists of two different layers (P and S) with different fibril positions resulting in different textures and densities (Alén 2000, Klemm et al. 1998). In addition, 20 – 30% of the wood material comprises hemicelluloses, which are low DP heteropolysaccharides (Jääskeläinen, Sundqvist 2007), located in the spaces between microfibrils (Sjöström 1993). The cell wall contains also lignin, which is a random branched polymer composed of aromatic phenylpropane units.

The outer layer of the cell wall is called the primary wall (P), which is about 0.05 – 0.1 μm thick and the microfibrils are arranged in a network-type of structure (Alén 2000, Klemm et al. 1998). The secondary wall (S) has three different layers S1, S2, and S3. The outer S1 layer of the secondary wall has a crossed fibrillar structure alternately to the left or right, with left being the dominant microfibrillar orientation. The average helix angles of the microfibrils are between 50° – 70° in the S1 layer. The thickness of the S1 layer is 0.1 – 0.3 μm and the number of microfibrillar layers is 3 – 6. The S2 layer of the secondary wall contains most of the cellulose mass and, to a large extent, dictates the physical properties of a wood fiber. The S2 layer is usually 1 – 8 μm thick and the number of microfibrillar layers is between 30 and 150. The S2 layer microfibrils have a high degree of parallelism and are orientated in a right-handed conformation with the average angles between 5° – 30°. The microfibrils in the S3 layer are gently sloped with the average angle of the microfibrils being 60° – 90°. The S3 layer is less than 0.1 μm thick and the number of microfibrillar layers is less than six. The central cavity of the fiber is called the lumen (L) which is coated with a wax. Middle lamella (ML) is located between the P walls of adjacent cells and functions as an adhesive layer of the cells. It is approximately 0.2 – 1.0 μm thick with high lignin content.

2.2.2 Nanocellulose

In general, cellulose nanoparticles are described as cellulosic materials with at least one dimension in the nanoscale (Moon et al. 2011). The nomenclature used to describe nanocellulosic materials has not been standardized and consequently there are inconsistencies in the use of the terms. However, recently the term cellulose nanofibrils (CNF) has been established in the literature. Nanoscale cellulose materials have been observed to display a different behavior and functionality when compared to the larger cellulosic fibers. The unique tensile, optical, electrical, and chemical properties of nanofibers in comparison with macroscopic cellulosic fibrous material are the result of their unique phys-
The main nanocellulose material utilized in this doctoral study is cellulose nanofibrils (CNF) but other types of nanocellulose are available, including cellulose nanocrystals (CNC) and bacterial cellulose (BC) (Figure 7).

Cellulose nanocrystals are obtained through acid hydrolysis of wood or plant fibers (Figure 7a), microcrystalline cellulose, MFC (Figure 7b) or CNF (Hubbe et al. 2008, Habibi et al. 2010). These rod-like or whisker-shaped nanoparticles (Figure 7c) have a high degree of crystallinity (54 – 88 %) and high aspect ratio, as they are commonly 3 – 5 nm wide and 50 – 500 nm long. In addition, they are almost pure cellulose and the dominating morphology is cellulose Iβ. Other labels used for cellulose nanocrystals include e.g. cellulose whiskers and nanocrystalline cellulose.

Certain bacteria from genera, such as Acetobacter, Agrobacterium, Alcaligenes, Pseudomonas, Rhizobium, or Sarcina are able to extracellularly secrete cellulose fibers (Figure 7d) with Gluconacetobacter xylinus (previously, Acetobacter xylinum) considered to be the most efficient synthesizer of bacterial cellulose (BC) (Iguchi et al. 2000, Siró, Plackett 2010). BC is free of lignin, hemicelluloses and from other biogenic products. Compared to plant fibers, BC has
higher crystallinity, higher water absorption capacity, better mechanical wet strength, is moldable, has ultrafine network structure, and is available initially in the wet state (Klemm et al. 2001). BC is secreted through cellulose biosynthesis as ribbon-shaped fibrils less than 100 nm in width, which contain finer nanofibrils with a width of 2 – 4 nm (Siró, Plackett 2010).

*Cellulose nanofibrils*

Cellulose nanofibrils (CNF) are renewable, biodegradable, hydrophilic, and flexible and they exhibit high aspect ratio and large surface areas (Siró, Plackett 2010, Iwamoto et al. 2008). CNF is reminiscent of the elementary fibrils in wood and plant cellulose biosynthesis (Moon et al. 2011, Pääkkö et al. 2007). CNF has a high aspect ratio due to its dimensions of 4 – 20 nm in width and 0.5 - 2 μm in length, consists almost entirely of cellulose, exhibits the Iβ or Iα crystal structure and contains both crystalline and amorphous regions. Due to non-standardized nomenclature, CNF is also sometimes referred to as microfibrillated/microfibrillar cellulose (MFC) and nanofibrillated/nanofibrillar cellulose (NFC) (Siró, Plackett 2010, Moon et al. 2011). However, in some cases MFC can be thought of as micrometer sized particles that consist of several elementary fibrils with larger dimensions compared to CNF (10 – 100 nm in width and 0.5 – 10 μm in length).

CNF is typically produced from delignified wood fibers by mechanical disintegration (Turbak et al. 1983) with or without chemical or enzymatic pretreatments. Mechanical disintegration extracts long cellulose fibrils along the longitudinal axis of the microfibrillar structure by producing high shear forces, which induce transverse cleavage. However, due to the interfibrillar hydrogen bonding and complex multilayer structure of fibers, nanofiber aggregates with a broad width distribution are commonly attained with the mechanical methods (Abe et al. 2007). Additionally, the mechanical deconstruction reduces the DP, lowering the molar mass of the microfibrils and the energy consumed by the process is high. Therefore, pretreatment methods, such as enzymatic (Pääkkö et al. 2007), (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO) radical mediated oxidation (Saito et al. 2006) or carboxymethylation (Wågberg et al. 2008) are applied to ease the fibrillation process. The enzymatic treatment does not alter the surface chemistry of the fibrils yielding nanocellulose with similar properties as the raw material whereas most prominent chemical pretreatment methods (TEMPO-oxidation and carboxymethylation) increase interfibrillar repulsion and swelling by introducing surface charges as anionic groups, easing the mechanical disintegration and also facilitating the isolation of nanofibrils, with controlled and uniform diameter distribution. Enzymatic pretreatment reduces bonds between cellulose nanofibrils through hydrolysis reactions catalyzed by cellulases, reducing energy consumption in the mechanical disintegration.

As the amorphous regions remain mainly intact during preparation, the degree of crystallinity of CNF is typically low (Pääkkö et al. 2007). CNF has a tendency to form highly disordered and entangled networks and gels in water, even at very low concentrations due to the high aspect ratio, allowing for entanglements and junction points for bonding. CNF aqueous suspensions also display shear-thinning rheological behavior (Klemm et al. 2011). Additionally, cellulose
nanofibrils have a small coefficient of thermal expansion (CTE), at $0.1 \times 10^{-6}/K$ (Iwamoto et al. 2008). Cellulose nanofibrils have also been found to have low toxicity for living organisms (Bhattacharya et al. 2012). The strength of a single fibril is reported to be 2-6 GPa (Saito et al. 2013) and the modulus in the range of tens of GPa (Iwamoto et al. 2009) due to the densely hydrogen bonded structure. These properties offer unique advantages and possibilities for CNF utilization in diagnostic and biomedical applications.

### 2.2.3 Cellulose functionalization

The chemical modification of cellulose nanofibrils can be incorporated together with their extraction, as is the case in carboxylation with TEMPO (Saito et al. 2009) and carboxymethylation (Wågberg et al. 2008, Karabulut et al. 2012). The chemical modification methods of cellulose nanofibrils also include acetylation (Rodionova et al. 2011, Tingaut et al. 2010), silylation (Andresen et al. 2006, Goussé et al. 2004, Xhanari et al. 2011, Qu et al. 2012) and cationization (Olszewska et al. 2011).

**TEMPO-oxidation**

In TEMPO-mediated oxidation the C6 primary alcohol groups in cellulose are selectively oxidized to carboxyls with a sequential reaction of the TEMPO-radical and an oxidant such as hypochlorite with NaBr as an additional catalyst at pH 10-11 (Saito, Isogai 2006). This method provides the CNF surface with negatively charged carboxylic acid groups i.e. produces β-1,4-linked polyglucuronic acid and the fibrils are easily dispersed with low speed mechanical treatment. An illustration of the proposed TEMPO-mediated oxidation reaction of cellulose is presented in Figure 8. In order to reduce the alkaline-induced decrease in cellulose degree of polymerization, the reaction has been developed to occur also at neutral or only slightly alkaline condition (TEMPO/NaClO/NaClO₂) (Isogai et al. 2011). Moreover, oxidation at neutral conditions reduces the amount of aldehydes on the cellulose material, which are otherwise present when alkaline conditions are utilized.
Figure 8. TEMPO/NaBr/NaClO regioselective oxidation of C6 primary hydroxyls of cellulose to C6 carboxylate groups in aqueous solution of pH 10–11 (Isogai et al. 2011).

Carboxymethylation
Carboxymethylation of cellulose (solvent-exchanged to ethanol) is conducted via etherification with monochloroacetic acid in organic solvents in the presence of NaOH. Carboxymethylation of fibers to low DPs was first investigated by Walhecka (1956) but was demonstrated as a pretreatment method for CNF preparation by Wågberg et al. (2008). The carboxymethyl group can be substituted to C2, C3 and C6 depending on the degree of substitution. Carboxymethylation also increases the negative charge of cellulose nanofibrils causing fiber swelling and thus they are disintegrated more easily from the macrofibrillar structure due to more pronounced electrostatic repulsion. Adsorption of carboxymethyl cellulose (CMC) onto wood fibers prior to fluidization has also been applied as a facile pretreatment method to produce CMC-modified CNF (Junka et al. 2014).

Other functionalization methods
The disintegration of nanofibrils from dissolving grade fibers can be performed with a cationization pretreatment followed by high-pressure homogenation (Olszewska et al. 2011, Aulin et al. 2010). This method yields cationic CNF. Improved adhesion with hydrophobic matrices is the goal in acetylation (Kim et al. 2002) where hydrophilic CNF is hydrophobized by acetic anhydride (AA) reaction with the hydroxyl groups on the cellulose molecules (Rodionova et al. 2011, Tingaut et al. 2010). Also maleic and succinic acid groups have been introduced to CNF surfaces via reactions with surface hydroxyl groups of cellulose and succinic or maleic anhydride (Stenstad et al. 2008). Surface silylation also allows for the formation of hydrophobically modified CNF (Goussé et al. 2004, Andresen et al. 2006). After silylation, the fibrils display a hydrophobic surface and thus become dispersible in solvents of low polarity while flocculate in aqueous media.
2.2.4 Nanocellulose products

CNF nanopapers/films

CNF has the ability to pack into organized structures when the solvent is evacuated. Cellulose nanofibril films are prepared by casting, either on a filter membrane with vacuum filtering of the solvent or on a solid surface via solvent evaporation (Moon et al. 2011, Siró, Plackett 2010). Sometimes pressing is applied together with casting. Cellulose nanofibrils in the CNF film are held together by extensive interfibrillar hydrogen bonding. These are stiff and strong films with some remnant porosity due to gaps in the nanofibril network. Because the diameter of CNF is less than one tenth of the wavelength of visible light, CNF is free from light scattering resulting in translucent films if the fibrils are adequately packed (Abe et al. 2007, Nogi et al. 2009).

Nanocellulose films usually exhibit isotropic in-plane formation and are typically 25 – 100 μm thick (Moon et al. 2011). The reported densities for cellulose nanoparticle films vary between 0.8 – 1.5 g cm\(^{-3}\) (Klemm et al. 2011). The mechanical properties of CNF films are also interesting. Nanopapers made of CNF exhibit good mechanical strength (Taniguchi, Okamura 1998). The average DP of CNF correlates with the tensile strength, toughness, and strain-to-failure of nanopapers. The higher average DP contributes for higher values for the above-mentioned properties (Henriksson et al. 2008). The densely packed fibrils give the nanopapers superior barrier properties (Syverud, Stenius 2009, Österberg et al. 2013). Immersion in water drastically reduces the mechanical strength of unmodified nanopapers, which do not re-disperse due to presumably a densely packed structure and hornification upon drying (Henriksson, Berglund 2007). TEMPO-oxidation leads to deteriorated wet mechanical properties for CNF nanopaper as the increased charge of the material makes the film more susceptible to water. However, CNF nanopaper can also be TEMPO-oxidized after preparation to maintain the wet strength of CNF nanopaper, with following diverse functionalization strategies (Orelma et al. 2012a). Figure 9 includes some few properties of CNF nanopaper and regular paper.

![CNF nanopaper and Paper](image)

**Figure 9.** Images of CNF nanopaper and regular paper placed on a printed background.
Wet-spun CNF filaments
Recently, a new type of cellulose filaments have been prepared from native cellulose nanofibrils via wet-spinning (Lundahl et al. 2017, Clemons 2016). In the wet-spinning process, a viscous solution or dispersion of polymer or spinning dope is forced through a needle or a spinneret to produce fibers or filaments. After leaving the needle, they are submerged in a coagulation bath or anti-solvent where the dope’s solvent is removed by chemical reaction or diffusion. The yielded filaments are subsequently dried to remove residual solvent. When the filaments are subjected to drawing, a high degree of molecular alignment can be achieved contributing to higher stiffness and strength of the material.

The prevailing technique to produce man-made cellulose fibers is the spinning of viscose. This involves laborious process steps and e.g. regeneration in sulfuric acid. In contrast, wet-spinning of CNF maintains the native cellulose I structure, which could attribute to better physical properties, such as higher stiffness and strength as compared to regenerated cellulose fibers. CNF wet-spinning also allows for more flexibility in shape selection for the resultant spun fibers. The needle parameter and speed of the process as well as the anti-solvent have an impact on the properties of the resulting filaments (Lundahl et al. 2017). Diverse morphologies have been observed for CNF filaments in the few studies on the topic. They include TOCNF filaments obtained from wood fibers presenting smooth surfaces, depending on spinning rate and tunicate-derived TOCNF filaments with circular cross-sections, rough and porous surfaces (Iwamoto et al. 2011) as well as others, such as nanoporous filaments with creasing along the axis (Walther et al. 2011, Lundahl et al. 2016). The setup of the wet-spinning process is presented in Figure 10.

![Figure 10. Schematic illustration of the wet-spinning process (Adapted from Tamayol et al. 2013).](image)

Wet strength of nanocellulose
The strength of nanocellulose films and filaments and the transferring of the mechanical properties of individual fibrils is dependent on the junctions of the CNF forming the network structure. The interfibrillar interactions are mainly governed by hydrogen bonding, which upon hydration are disrupted by immersion in water, resulting in significant loss of mechanical strength (Benítez et al. 2013, Toivonen et al. 2015). These effects become even more pronounced if carboxylation, such as TEMPO-oxidation is involved in the easing of the fibrillation...
process to reduce energy consumption as anionic carboxylate groups are evenly and regularly present on the fibril surfaces leading to osmotic pressure and electrostatic repulsion between individual fibrils in aqueous environment (Okita et al. 2010). The susceptibility to water is a limiting factor in utilizing nanocellulose-based materials in application requiring humid or aqueous conditions. Therefore, recent efforts have been focused on improving the wet strength of CNF products.

The strategy to improve the wet mechanical properties of cellulose nanofibrils products can involve covalent crosslinking. Recently, glutaraldehyde was utilized to covalently crosslink wet-spun CNF filaments to increase water-resistance (Mertaniemi et al. 2016). Heat-induced esterification between carboxyl groups of added poly(acrylic acid) and hydroxyl groups present in native CNF can enhance the wet strength of CNF films (Spoljaric et al. 2013, Caulfield 1994). CNF composite materials with increased wet strength have also been developed by impregnation in e.g. epoxy (Ansari et al. 2014), phenol (Qing et al. 2013, Nakagaito, Yano 2005) or melamine formaldehyde resins (Henriksson, Berglund 2007). Ionic crosslinking between multivalent ions and oxygen-containing groups of TEMPO-oxidized bacterial cellulose fibrils has also been employed to yield high mechanical strength wet-spun filaments that in humid conditions retain over 70% of the initial dry strength (Yao et al. 2017). Wet mechanical properties of casted CNF films have been shown to increase by physical crosslinking utilizing carbohydrate chemistry between CNF and chitosan (Toivonen et al. 2015). An emerging technique to improve CNF film water resistance is the partial dissolution method. Here prepared CNF films or nanopapers are impregnated with aqueous solution of N-methylmorpholine-N-oxide (NMMO). The films are subsequently heat calendered to facilitate surface-sensitive dissolution resulting in reinforced matrix structure (Orelma et al. 2017).

2.2.5 Ultrathin cellulose model films

Fibers and fibrils exhibit complex surface structures due to material properties, such as chemical complexity, roughness and porosity, rendering difficult any attempt to study surface interactions. However, the development of soluble cellulose derivatives, cellulose dissolving solvents and nanocellulose facilitated the preparation of thin and ultrathin films that can function as model surfaces/films. Additionally, the emergence and popularization of many surface-sensitive techniques, such as atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS) and quartz crystal microbalance (QCM), have expanded the interest and information gained in model film studies. Model thin films also provide clearly defined chemistries and morphologies ideal for surface research (Kontturi et al. 2006, Roman 2009).

The cellulose-based model films can be utilized to mimic the chemistry of native cellulose fibers and fibrils. The first cellulose model films were prepared in 1972 (Agnihotri, Giles) by dissolving cellulose in cadoxen (tri(ethylenediamine) cadmium hydroxide) and casting a monolayer on water. Since then different cellulose model films have been developed, including regenerated cellulose, cellulose nanofibril, cellulose nanocrystal and cellulose bilayer surfaces (Kontturi et
There are two main methods to prepare cellulose model films: spin-coating and horizontal Langmuir-Blodgett, i.e., Langmuir-Schaef er deposition. Both these methods utilize dissolved or dispersed material in liquid medium for depositing an ultrathin film. The deposited thickness of the ultrathin cellulose film is 5 – 100 nm. It is important that an evenly covering layer of cellulose is deposited on the support material, typically 15 nm is enough to achieve this.

In the Langmuir-Schaef er method, a hydrophobic monolayer of dissolved substance is prepared on top of water contained in a trough. The properties of the hydrophobic monolayer are controlled with the surface pressure, and a hydrophobic support is dipped onto the water surface. Theoretically, the hydrophobic monolayer on top of the water surface is then transferred onto the support. However, several dips are typically required to obtain uniform films. Cellulose can be derivatized prior to deposition to facilitate its dissolution in organic solvents by e.g. hexamethyldisilazane. Trimethylsilyl cellulose (TMSC) can be applied to prepare cellulose II model films by the LB and LS methods (Tammelin et al. 2006). The deposited TMSC can be converted back to cellulose by HCl vapor treatment (Schaub et al. 1993, Kontturi et al. 2003). Very thin (0.5 nm) cellulose II surfaces with high crystallinity (60%) can be prepared with this method (Aulin et al. 2009).

Relevant to the present work are spin-coated cellulose films. Spin-coating is a relatively simple method for ultrathin film preparation, where a drop of polymer solution is placed on a solid support which is then rotated at a certain angular velocity for a given time period (Amim et al. 2008). Excess polymer solution is thrown off the substrate due to its high angular velocity and only a small layer of solution remains. Subsequently, a uniform thin layer is formed as the solvent evaporates leaving only a polymer layer on top of the substrate (Kontturi et al. 2003). TMSC can be spin-coated onto hydrophobized supports yielding highly amorphous cellulose II films upon HCl treatment, yielding films with a roughness of ~3 nm. Native cellulose I nanofibrils dispersed in water can also be spin-coated onto a supporting material. Model films with ~3.2 nm roughness can be obtained with this method. CNF films have also found utilization as model films representative of the outer surface of cellulose fibers (Ahola et al. 2008b). Figure 11 exhibits AFM images of typical spin-coated regenerated cellulose and CNF model films.
2.3 Proteins and immunoassay development

Biomolecules play an important role in developing diagnostic and biomedical applications as they function as e.g. biorecognition agents or biosensors (Pelton 2009). In the next section, protein structure and immobilization strategies are discussed together with material requirements for biosensor development.

2.3.1 Protein chemistry

Proteins are macromolecules constructed from amino acids linked together by peptide bonds (Berg et al. 2012). Amino acids are composed of an \( \alpha \)-carbon connected to a carboxyl group, an amino group, a hydrogen atom and a side chain. Only L isomer amino acids are protein constituents. All proteins are constructed from a combination of up to 20 different amino acids. Based on their side chains, the amino acids can be classified into four categories: hydrophobic, polar, positively charged, and negatively charged. Figure 12 includes an illustration of protein folding patterns and different structural levels.

Figure 12. Schematic illustration of polypeptide chain folding to form the four levels of protein structures (adapted from Reece et al. 2011).
Peptide bonds take place between the \( \alpha \)-amine groups and \( \alpha \)-carboxyl groups of the amino acids (Berg et al. 2012). The linked amino acids are called polypeptides and form the primary structure of proteins. The amino acid units in a polypeptide are called residues. The polypeptide chain also has polarity as the other end contains an \( \alpha \)-amine (N-terminus) and the other end an \( \alpha \)-carboxyl (C-terminus) (Reece et al. 2011, Berg et al. 2012). By convention, the amino acid sequence of a protein is given from the N-terminus to the C-terminus. The secondary structure of proteins is formed when the NH and CO groups that are near to each other in the linear polypeptide backbone form regular patterns of hydrogen bonds. The result of this folding are rod-like structures called \( \alpha \) helices where the coiled polypeptide backbone forms the inner part and the side chains extend outward in a helical array. Other secondary structures are sheet-like formations called \( \beta \) sheets where extended \( \beta \) strands of two or more polypeptide chains are linked through hydrogen bonding, forming a planar structure. Reverse turns and loops are secondary structures responsible for stabilizing abrupt changes in the direction of polypeptide chains and for more elaborate chain reversals, respectively. Long-range interactions between amino acid residues are responsible for the tertiary structure of proteins. Typically, non-polar residues fold into the interior of protein and polar, charged side chains arrange on the surface. Multiple polypeptide chains can assemble into multiple subunit structures displaying a quaternary structure. Some treatments, such as heating, disrupt the tertiary structure of the protein.

**Antibodies**

Protein molecules that have specific interaction with antigenic determinants are called antibodies or immunoglobulins. There are five major classes of immunoglobulins, IgG, IgA, IgM, IgD, and IgE, based on their different physical, chemical, and immunological properties as well as their behavior as antigens (Madigan et al. 2009, Elgert 1996). About 80 % of the human serum antibodies are Immunoglobulin G (IgG) proteins. The IgG structure contains two identical heavy chains and two identical light chains containing ~440 and ~220 amino acids, respectively. Immunoglobulin G is able to bind two antigens as each heavy chain interacts with a light chain to create a functional binding site. The chains of IgG are composed of domains that are linked with disulfide bonds. In the heavy chains a variable domain (\( V_H \)) is connected to three constant domains (\( C_H1, C_H2, C_H3 \)) and in light chains there are two domains, a variable (\( V_L \)) and a constant (\( C_L \)) domain. The constant domains in all IgG proteins are identical and the variable domains determine the specific antigen binding as the amino acid sequences in variable domains differ in each antibody. An illustration of the structure of IgG is presented in Figure 13.
The IgG molecule can be described also by how the antibody functions are located (Madigan et al. 2009). The fragments, consisting of the light chain and half of the heavy chain, are responsible for antigen recognition and are called Fab fragments. The fragment containing the rest of the heavy chains is called Fc region. The variable domains in the Fab fragments bind the antigen based on their variable amino acid sequence specific to each type of antibody. The dimension for the Y-shaped Immunoglobulin G are reported to be 14.5 nm in height, 8.5 nm in width and 4.0 nm in thickness (Silverton et al. 1977, Lee et al. 2002).

**Antibody – antigen interaction**

As discussed earlier the variable domains of the heavy and light chains form together the antigen-binding site. The amino acid sequences in variable domains in different antibodies differ greatly, particularly in several complementarity-determining regions (CDR) (Madigan et al. 2009). Both, the variable domain of the heavy and light chains contain three CDRs. The antigen binding of the antibody is essentially determined by the folding of the heavy and light chain variable domains bringing all the six CDRs together at end of the antibody, resulting in a unique and specific binding site. Several mechanisms, such as somatic recombination, random heavy- and light-chain re-assortment and hypermutation, in antibody generation in B cells contribute to the fact that almost limitless diversity of different antigen-binding site on antibodies can be produced, as antibodies are required to recognize and bind a wide variety of molecular structures.

The antigen-binding site of an antibody, approximately 2×3 nm, is large enough to accommodate a small portion of the antigen called an epitope, which is about 10-15 amino acids long. Common types of epitopes include sugars, short peptides and other organic molecules. Antibodies are able to distinguish even between very similar epitopes, such as glucose and galactose (orientation of single hydroxyl group).
2.3.2 Protein immobilization

The solubility of proteins in water is strongly facilitated by the numerous dissociable groups they contain (Böhme, Scheler 2007). Furthermore, there is always a pH-dependent balance of negative and positive charges in proteins as they contain both acid and amino groups. The charge observed from a distance is the effective charge of the protein and is responsible for the protein’s behavior in external electric fields. The functional groups of proteins originate from the different amino acid side chains (Rusmini et al. 2007). The commonly available functional groups are amino groups from lysine and hydroxyl-lysine, thiol groups from cysteine, carboxylic acid groups from aspartate and glutamate, and hydroxyl groups from serine and threonine.

There are three main immobilization methods of proteins on surfaces (Rusmini et al. 2007). In physical immobilization, intermolecular forces, such as ionic bonds or polar and hydrophobic interactions, facilitate physical adsorption of proteins on different surfaces. The physical adsorption of proteins on surfaces is influenced by solution conditions such as pH and ionic strength and proteins may experience conformational changes upon adsorption (Norde 2008). Covalent immobilization involves the covalent binding of proteins on a (suitably modified) support via the accessible functional groups of the exposed amino acids (Rusmini et al. 2007). Bioaffinity immobilization method of proteins utilizes the biochemical affinity of certain molecules such as the specific avidin–biotin interaction. Opposed to the other methods, bioaffinity attachment allows for oriented and homogenous attachment of proteins on surfaces.

Physical adsorption

Physical adsorption is a relatively straightforward method for coating surfaces via non-covalent interactions, such as van der Waals forces, electrostatic forces, hydrophobic interactions, or hydrogen bonding. However, molecules attached via physical interactions can be relatively loosely adhered to the surface and form a randomly oriented, heterogeneous surface with reduced activity (Kong, Hu 2012, Rusmini et al. 2007). In addition, the density of the resultant surface may be low. For example, upright conformation and controlled surface coverage promote better functionality for DNA assays (Lee et al. 2006, Schreiner et al. 2010).

Many biomedical applications require the saturation or immersion of the material in water. In water, the fiber or fibrils become a swollen hydrogel of cellulose and hemicelluloses with some anionic charge (Pelton 2009). Therefore, colloidal particles, non-ionic and negatively charged water-soluble polymers (Wågberg, Ödberg 1989, Hendrickson, Neuman 1986) in addition to DNA (Su et al. 2007) have minimal tendency to adsorb on fiber surfaces. Often, modification of the cellulose surface is in order as only cationic particles and molecules tend to adsorb.

The adsorption of proteins on cellulosic materials is affected by electrostatic interactions (Jones, O’Melia 2000, Karra-Châabouni et al. 2008). Proteins may also experience conformational changes upon adsorption (Norde 2008). Cat-
onic polymers or cationic patches of proteins adsorb to anionic cellulose surfaces. Some evidence indicates that protein adsorption is also influenced by interaction of cellulose with the tyrosine groups (Lehtio et al. 2003, Mattinen et al. 1997). The properties of cellulose and proteins are also susceptible to pH, ionic strength, and specific ion effects. The immobilization of proteins onto wood fibers probably requires special protocols since proteins are not strongly adsorbed to cellulose (Brash, Ten Hove 1993).

The difficulties of physical adsorption include the low affinity of proteins, phages, and DNA aptamers to cellulosic material. If the cellulose surface is modified with cationic polymers, such as PAE to increase adsorption, the highly reactive cationic surfaces tend to denature proteins due to unfolding. As most biomolecules adsorb on cationic surfaces, non-specific adsorption needs to be prevented with blocking agents (Reimhult et al. 2008), such as BSA, non-ionic surfactants, casein or fat-free milk (Vikholm-Lundin 2005). One approach to physical adsorption of proteins to cellulose is layer-by-layer (LbL) deposition (Decher, Schlenoff 2012). The LbL assembly is typically electrostatically driven (Zhang et al. 2007) and can be utilized to embed biomolecules, such as viruses (Yoo et al. 2006) and enzymes (Xing et al. 2007) onto surfaces. Orelma et al. (2011) have demonstrated that the adsorption of bovine serum albumin (BSA) and human immunoglobulin G (hIgG) is dependent on pH and the charge of the cellulose surface.

### Chemical immobilization

The most stable immobilization method is covalent chemical bonding. In this approach, biomolecules are linked to the cellulosic material via functional chemical groups (Kong, Hu 2012). Covalent bonds are typically formed via the exposed side-chain functional groups of proteins (Rusmini et al. 2007). These include amine groups originating from lysine residues, thiol groups from cysteine residues, carboxyl groups from aspartate and glutamate residues and hydroxyl groups from serine and threonine residues. Covalent immobilization results in irreversible attachment and high surface coverage. Covalent protein attachment can be divided to non-specific and site-specific immobilization (Steen Redeker et al. 2013).

Non-specific immobilization strategies include amine, thiol, carboxyl, epoxy, and photoreactive chemistry (Rusmini et al. 2007). Surfaces containing maleimide, pyridyl disulfide or vinyl sulfone groups can react with thiol groups originating from cysteine to form covalent bonds. Epoxy chemistry usually utilizes a two-step approach of adsorption and covalent immobilization as covalent reactions between epoxy groups and proteins are very slow and require fast adsorption followed by intramolecular chemical attachment. Photoreactive chemistry requires the presence of photosensitive reagents, such as arylazides, diazirines, benzophenones, or nitrobenziles for immobilization. Covalent bonding is induced by light activation. This fast and efficient route does not require activation chemicals.

N-hydroxysuccinimide (NHS) is the most commonly applied coupling agent with amine groups for formation of amide bonds (Rusmini et al. 2007, Steen Redeker et al. 2013). Amine groups from lysine residues are typically present on
the exterior of the protein and therefore make good anchoring points with surfaces exhibiting carboxyl, aldehyde, epoxy groups or active esters. NHS is often applied together with (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (EDC) to activate carboxylic groups for protein coupling (Figure 14). Due to the low nucleophilicity of carboxyl groups in water, covalent bonding does not occur spontaneously and activators are needed (Hermanson 2008). EDC (Grabarek, Gergely 1990) and carbonyldiimidazole (CDI) (Fernandez-Lafuente et al. 1993) are the most common coupling agents utilized with carboxyl groups but because of the water-solubility of EDC it has been widely utilized for protein conjugation. An active O-acylisourea intermediate is formed when EDC reacts with the carboxyl anion. This intermediate is easily displaced by amine-containing molecule by nucleophilic attack (Figure 14). However, due to the instability of the O-acylisourea intermediate, if the reaction does not occur within seconds with the amine-containing molecule, the N-unsubstituted urea is released into the medium (Gilles et al. 1990, Nakajima, Ikada 1995) (Figure 14). Addition of NHS in the reaction mixture stabilizes the system as it converts the amine-reactive intermediate to an amine-reactive NHS ester. This NHS-ester intermediate is semi-stable in water and significantly increases the amide bond formation (Staros et al. 1986) (Figure 14). EDC/NHS activation was the main method employed in this work to crosslink molecules onto cellulose.

Site-specific covalent immobilization include techniques, such as “click” chemistry where crosslinking of azide- and alkyne-functionalized entities to form 1,2,3,-triazole bonds. Also, in Diels-Alder cycloaddition immobilization electronically matched dienophile and conjugate diene form a six-membered unsaturated ring (Steen Redeker et al. 2013). Staundinger ligation where an

![Figure 14. Schematic representation of the EDC/NHS chemistry with alternate pathways presented as well.](image-url)
aza-ylide is produced by reaction of azides with triphenylphosphines, photoactivatable thiol-ene chemistry, oxime ligation with oxyamine or hydrazide and aldehyde or ketone are also site-specific covalent immobilization methods.

Chemical immobilization of biosensors to cellulosic materials has been demonstrated (Bora et al. 2006, Dikmans et al. 2006, Tiller et al. 2002, Bryjak et al. 2008, Tyagi et al. 2009, Kong et al. 2002, Reinhartz et al. 1993, Bartouilh de Taillac et al. 2004, Goldstein et al. 1990, Credou et al. 2013). Cellulosic materials exhibit abundant surface hydroxyl groups that can be exploited for conjugation. Typical activation reactions of cellulose include the surface hydroxyl group functionalization via periodate oxidation (Van Leemputtes, Horisberger 1974), via epoxide chemistry (Uy, Wold 1977) and via reaction with CDI (Stöllner et al. 2002). Also cellulose architecture has been modified to allow for the immobilization of biomolecules by reacting p-toluenesulfonic acid esters of cellulose with 1,4-phenylenediamine (PDA) to gain redox-chromogenic properties and anchor groups (Tiller et al. 1999). The hydroxyl reactive chemistries require at least one step in non-polar solvents as the hydroxyl groups of cellulose are too unreactive for specific reactions at low temperatures in water (Kong, Hu 2012). Thus, cellulose needs to be functionalized or derivatized with small molecules or polymers to achieve surface functionality that allows for bioconjugation. The biomolecules and cellulosic support material are bridged by homo- or heterobifunctional chemical linkers (Su et al. 2007, Bora et al. 2006, Tiller et al. 2002, Kong et al. 2002, Wu, Lia 2008). Alkaline phosphatase chemical conjugation by epoxy, amine, and TEMPO-oxidation functionalized CNF has been demonstrated (Arola et al. 2012). Additionally, EDC/NHS chemistry has been employed to conjugate proteins onto carboxylated cellulose surfaces (Orelma et al. 2012c, Orelma et al. 2012b). Cellulose modification with the copper (I)-catalyzed 1,3-dipolar cycloaddition of alkynes and azides (CuAAC) has been also demonstrated in modification of cellulosic materials (Filpponen, Argyropoulos 2010, Filpponen et al. 2012, Junka et al. 2014).

Bioaffinity attachment

Bioaffinity attachment allows for oriented and homogenous attachment of proteins on surfaces. Bioaffinity immobilization method of proteins utilizes the biochemical affinity reactions of certain molecules such as the specific avidin – biotin interaction (Green 1975) or protein A interaction with IgG (Deisenhofer 1981). The conformation of conjugated proteins can be controlled also with different chemistries, such as immobilization of liberated Fab fragments with sulfide bonds (Lu et al. 1995), immobilization through carboxyl residues at C-termini of antibodies with carboxypeptidase (You et al. 1995) or the oxidized carbohydrate moieties of antibodies (O’Shannessy, Hoffman 1987).

Protein A is a covalently bound protein in the cell wall of *Staphylococcus aureus* that can be isolated or secreted (Hsu, Raine 1981, Graille et al. 2000). Protein A is capable of binding the Fc portion of most IgG molecules of various species without disrupting the antigen recognition capabilities of the antibody (Björk et al. 1972). Protein A has five highly homologous regions or domains designated as E, D, A, B, and C that are each able to bind to the Fc region of IgG.
(Graille et al. 2000). However, a disadvantage in utilizing protein A for immobilization of IgG molecules is the lack of control in the orientation of protein A itself so that the binding sites remain free after immobilization (Rusmini et al. 2007).

Avidin is a tetrameric glycoprotein with the ability to bind up to four biotin molecules. Avidin – biotin bond formation is very rapid and described as one of the strongest non-covalent bonds known, with a dissociation constant of $K_D = 10^{-15}$ M (Green 1975, Luo, Walt 1989). Biotin, also known as vitamin H, is a small molecule found in all living cells. The bicyclic ring of biotin interacts with avidin and this allows for the modification of the carboxyl group of the valeric acid side chain to generate biotinylated reagents for protein conjugation. A common approach for avidin attachment is to create a multilayer by first immobilizing biotin on a surface followed by creation of an avidin layer that can bind another layer of biotinylated molecules (Rusmini et al. 2007, Sun et al. 2002). This is a popular approach since it yields higher organization as opposed to direct avidin immobilization on surfaces as the pre-immobilized biotin layer promotes avidin to have an ordered structure where two binding sites for biotin face the surface and the two other sites face outward. Orelma et al. (2012b) have demonstrated that avidin either adsorbed or chemically conjugated via EDC/NHS activation directly on carboxylated cellulose can be utilized in attaching oriented biotinylated functionalities on cellulose.

Other bioaffinity attachment methods include utilization of polyhistidine as an affinity tag or His-tag where histidine-rich regions of protein interact with divalent metal ions (Steen Redeker et al. 2013, Rusmini et al. 2007). DNA-directed immobilization involves the conjugation of proteins with artificial nucleic acids. Affinity capture ligand systems involve the fusions of proteins of interest with other proteins that chemoselectively react with substrates present on surfaces. Carbohydrate chemistry can also be exploited in attaching glycoproteins as the carbohydrate region of proteins rarely is involved in specific activity.

Bioaffinity attachment on cellulose can also involve the utilization of cellulose binding domains (CBD) or molecules (CBM) on enzymes or other biochemical binding agents as suitable tags for affinity to cellulose (Kong, Hu 2012, Pelton 2009, Linder et al. 1998). When CBMs are fused with the biomolecule to be immobilized, such as llama antibodies or protein A, the attachment to cellulose is relatively simple (as the CBMs spontaneously bind to cellulose) (Lewis et al. 2006, Cao et al. 2007). CBMs have been successfully utilized in cell (Craig et al. 2007) and antibody (Cao et al. 2007) attachment as well as in immobilization of bacteriophages and enzymes (Tolba et al. 2010). There are also reports on utilizing CBMs with streptavidin to attach biotinylated TiO$_2$ on cellulose (Ye et al. 2007) and of cellulose-binding DNA aptamers (Boese et al. 2008).

### 2.3.3 Controlling non-specific binding

Biomedical applications typically require controlled immobilization of recognition molecules on the substrate, but also anti-fouling properties for lowering non-specific biomolecule adsorption to minimize interference (VandeVondele...
et al. 2003). Such non-specific adsorption is problematic since it may block otherwise active binding sites or result in a false positive bioassay (Rusmini et al. 2007). Protein-resistant surfaces are also important in developing implantable systems to reduce e.g. rejection and fouling (Vaddiraju et al. 2010). Prominent methods to minimize non-specific protein adsorption include different protein-resistant coatings with polymers such as dextran (Holland et al. 1998), polyvinyl alcohol (Amanda, Mallapragada 2001) and polyacrylamide (Park et al. 2000). Moreover, blocking agents widely utilized against non-specific protein and bacteria adsorption include macromolecules based on polyethylene glycol (PEG) (Scott, Murad 1998, Huang et al. 2001, Pasche et al. 2003, VandeVondele et al. 2003, Maddikeri et al. 2008, Islam et al. 2014a). However, it is established that PEG alone has low affinity for cellulose (Filpponen et al. 2012, Holappa et al. 2013) but various indirect routes mediate their interactions (Wang et al. 2006, Olszewksa et al. 2013, Deng et al. 2014).

A promising method to gain minimal and reproducible non-specific adsorption involves the installation of multifunctional polymers incorporating surface-binding and surface-passivating domains (VandeVondele et al. 2003, Pasche et al. 2003, Zhang et al. 2012a, Zhang et al. 2013a). Block polymers with PEG blocks have been shown to passivate hydrophobic substrates (Neff et al. 1998), whereas cationic poly-(L-lysine) grafted with multiple PEG side chains has yielded a similar effect for negatively charged metal oxide surfaces (VandeVondele et al. 2003, Huang et al. 2001, Pasche et al. 2003).

### 2.3.4 Requirements for CNF-based biomedical applications

As discussed previously, in order for CNF-based materials to find utilization as advanced, high-efficiency platforms in biomedical engineering they must meet certain material criteria. Due to the relative inertness of the hydroxyl groups of cellulose, different functionalization methods are typically required for biomolecule attachment. However, these functionalization strategies may come at a cost of other characteristics and impair the mechanical performance in aqueous environments, as is the case with TEMPO-oxidation and carboxymethylation. Therefore, rendering the material adequately water-resistant is an essential requirement. Biomedical application typically also requires antifouling properties of the surface to allow for controlled biomolecule attachment, free from interferences and for accurate biosensor development. Desirable would also be the possibility of regeneration of the material for multiple testing cycles. Figure 15 illustrates the characteristics required of CNF materials for diagnostic purposes.
The World Health Organization (WHO) has implemented a set of criteria for diagnostic platforms called “ASSURED”. The platforms should be affordable, sensitive, specific, user-friendly, rapid, equipment-free, and deliverable to the users (Mabey et al. 2004). Nanocellulose systems might prove to be too complex for traditional type of rapid diagnostics but offer interesting opportunities for the development of emerging biomedical applications. However, as the cellulose-based paper and thread diagnostic systems have recently gained increased attention, it is also important to characterize the interactions between biomolecules and cellulosic substrates as well as to tune the material properties for more efficient operations. Relevant to this work is the fact that cellulose nanofibrils facilitate studies in the form of films and open possibilities for surface-sensitive studies of related interactions.
3. Experimental

The work carried out in this thesis was focused on surface modification of cellulose nanofibrils for utilization as bioactive materials. The research is to large extent centered on cellulose-based ultrathin films. The model films applied in this work were spin-coated regenerated (amorphous) cellulose (Paper I), spin-coated CNF (Paper I), spin-coated and in situ TEMPO-oxidized CNF (TOCNF, Papers I and II) and spin-coated benzophenone-functionalized, TEMPO-oxidized CNF (BP-CNCF, Papers III and IV). Other substrate materials utilized included nanopaper prepared from BP-CNCF (Paper III) and filaments of BP-CNCF obtained by wet-spinning (Paper IV).

The main fabrication and analytical methods utilized in this thesis were spin-coating (Papers I-IV), nanopaper preparation via pressure filtration (Paper III), surface plasmon resonance (SPR) (Papers I, II and IV) quartz crystal microbalance with dissipation (QCM-D) (Papers I and IV), atomic force microscopy (AFM) (Papers I-IV) and X-ray photoelectron spectroscopy (XPS) (Papers I-III).

3.1 Materials

3.1.1 Cellulosic materials

Regenerated cellulose
Trimethylsilylcellulose (TMSC) utilized in Paper I was synthesized by hexamethyldisilazane silylation of microcrystalline cellulose powder from spruce fibers dissolved in DMAc-LiCl (Müller, Beck 1978, Cooper et al. 1981, Greber, Paschinger 1981). The details of TMSC synthesis applied in this work can be found in Tammelin et al. (2006). TMSC was spin-coated (Model WS-650SX-6NPP, Laurell Technologies, PA, USA) onto silicon dioxide QCM-D sensors (Q-Sense, Västra Frölunda, Sweden) according to Kontturi et al. (2003). TMSC was converted to regenerated cellulose by vapor-phase acid hydrolysis treatment (Schaub et al. 1993, Kontturi et al. 2003).

Cellulose nanofibrils
Cellulose nanofibrils (CNF) were used as isolated (Paper I) or as a precursor for TEMPO-oxidized CNF model films (Papers I and II). CNF was produced by processing bleached sulfite birch fibers through a M110P fluidizer (Microfluidics corp., Newton, MA, USA) equipped with 200 and 100 μm chambers and operated at 2000 bar pressure (at least 12 passes were used). CNF model films were
spin-coated onto PEI-coated silicon dioxide or gold QCM-D sensors (Paper I) or gold SPR sensors (Oy BioNavis Ltd., Ylöjärvi, Finland) (Papers I and II) according to Ahola et al. (2008b). The CNF-gel was diluted (0.190 wt% CNF in water) and ultrasonicated for 10 minutes at 25 % amplitude setting and consecutively centrifuged at 10400 rpm for 45 minutes. The nanofibrils were obtained from the resultant clear supernatant and spin-coated on the sensors.

**TEMPO-oxidized cellulose nanofibrils**

TEMPO-mediated oxidation was utilized to introduce carboxyl groups onto cellulose nanofibrils in Papers III and IV. The oxidation of the hardwood fibers was performed according to Isogai and Kato (1998) with a solution of 0.13 mmol (2,2,6,6-tetramethyl-piperidin-1-yl)oxyl (TEMPO, Sigma-Aldrich, Finland), 4.65 mmol NaBr, and 5.00 mmol NaClO in water at pH 10. The reaction was stopped with ethanol and MilliQ-water rinsing. The resultant oxidized fibers were passed once through a M110P microfluidizer operated at 2000 bar pressure with 200 and 100 μm chambers. TEMPO-oxidized CNF (TOCNF) model films were utilized to study copolymer and protein adsorption. CNF model films were prepared similarly as described before but the TEMPO-oxidation process was monitored in real time in the QCM-D (Paper I) and SPR (Papers I and II) modules according to Orelma et al. (2012b). The resultant TEMPO-oxidized CNF model films were used for measurements in the SPR and QCM-D setups immediately without drying steps. Detailed descriptions of the in situ TEMPO-oxidation procedure are available in Papers I and II.

**Benzophenone-functionalized TEMPO-oxidized cellulose nanofibrils**

Preparation of benzophenone-activated TEMPO-oxidized cellulose nanofibrils (BP-CNF) is described in more detail in Paper III. Briefly, after TEMPO-oxidation, the fibers were diluted in deionized water/dimethyl sulfoxide (DMSO) (90/10%) with 0.1M EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), and 0.4 M NHS (N-hydroxysuccinimide). Benzophenone-cross-linked fibers were obtained after 4-aminobenzophenone (Sigma-Aldrich, Finland) was added to the dispersion and the reaction was allowed to proceed overnight. The resultant functionalized fibers were passed once through the M110P microfluidizer operated at 2000 bar pressure with 200 and 100 μm chambers, producing benzophenone-coupled nanofibrils, BP-CNF. BP-CNF model films (Papers III and IV) were prepared similarly as described above for spin-coated CNF films.

**CNF nanopaper**

The procedure for the preparation of the CNF nanopaper is described in more detail elsewhere (Österberg et al. 2012). In short, the films were produced by pressure filtration of the obtained BP-CNF and TOCNF aqueous suspensions through a filter membrane (SEFAR NITEX 03-10/2, mesh opening 10 μm) under 2.5 bar pressure (Paper III). Pressing was applied to further consolidate the wet CNF film structure with a smooth metal rolling pin for five times after which the films were wet-pressed for four minutes. The nanopapers were dried between clean blotting boards for 2 hours at 100 °C and 1800 Pa and stored at
ambient conditions. UV light at 356 nm wavelength with 16 J cm\(^{-2}\) activation energy (Uvitec CL 508L UV chamber, Cambridge, UK) was utilized for photocrosslinking.

**Wet-spun CNF filaments**

BP-CNF and TOCNF gels (2%) were wet-spun into an acetone coagulation bath at a speed of 7.5 m min\(^{-1}\) (10 ml min\(^{-1}\)) through a needle (length 10.5 cm, diameter 1.3 mm) to obtain filaments (Paper IV). After approximately 5 min in the coagulation bath, the produced filaments were air-dried. To prevent longitudinal contraction during air-drying, the filaments were suspended from both ends. The resultant dry BP-CNF and TOCNF filaments were exposed to UV light at 356 nm wavelength with 16 J cm\(^{-2}\) activation energy (Uvitec CL 508L UV chamber, Cambridge, UK).

### 3.1.2 Block and Random copolymers

Poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) and poly(oligo(ethylene glycol)methyl ether methacrylate) (POEGMA) block copolymers (Papers I and II) were prepared by reversible addition−fragmentation chain-transfer (RAFT) polymerization. Random copolymers of the same monomers (Papers I, II and IV) were prepared by atom-transfer radical-polymerization (ATRP). Detailed descriptions of the polymerization processes and characterization with size exclusion chromatography are provided in Paper I. The average number of ethylene glycol units per oligo(ethylene glycol)methyl ether methacrylate monomer was 8-9 and the block and random copolymers were quaternized with methyl iodide. The PDMAEMA- POEGMA copolymers are referred to as D\(_n\)-b-EG-MA\(_m\) (block) or D\(_n\)-rnd-EGMA\(_m\) (random), where \(n\) and \(m\) represent the number of DMAEMA and OEGMA units in the copolymer, respectively. A representation of DMAEMA and OEGMA with proposed structures for the block and random copolymers are presented in Figure 16.

![Figure 16](image-url). Schematic illustration of the molecular structure of 2-(dimethylamino)ethyl methacrylate (DMAEMA) and oligo(ethylene glycol)methyl ether methacrylate (OEGMA) monomers (a) and representative structure of PDMAEMA-POEGMA block (b) and random (c) copolymers.
3.1.3 Biomolecules

Biointerface preparation via antibody conjugation onto TOCNF (Paper II) and BP-CNF (Paper IV) was performed utilizing anti-human immunoglobulin G (anti-human IgG, anti-hIgG) and anti-human hemoglobin (anti-Hb) antibodies, respectively. Non-specific protein adsorption onto copolymer passivated and unmodified TOCNF and the functionality of the anti-human IgG biointerface in Paper II was studied with human IgG. Protein A from *Staphylococcus aureus* was utilized for oriented biointerface preparation onto TOCNF (Paper II). The functionality of the anti-Hb biointerface prepared on BP-CNF was investigated with hemoglobin. Anti-Hb antibody fluorescence labeling was carried out with dansyl chloride and fluorescein 5(6)-isothiocyanate (FITC), described in more detail in Paper IV. All of the above-mentioned biomolecules were obtained from Sigma-Aldrich (Finland). Detailed descriptions and specific concentrations of biomolecule experiments are available in Papers II and IV.

3.1.4 Other chemicals

In Paper II bovine serum albumin (BSA) and methoxypolyethylene glycol amine (PEG-amine) were purchased from Sigma-Aldrich (Finland) and utilized to study the passivation of TOCNF towards non-specific hIgG adsorption. Additionally, SuperBlock®, a commercial protein solution, was obtained from Pierce (Rockford, IL, USA) and utilized for the same purpose. All other laboratory chemicals in Papers I-IV were of analytical grade and used without additional purification. Deionized water further purified with a Milli-pore Synergy UV unit (MilliQ) was used in all experiments.

3.1.5 Conjugation chemicals

Conjugation of proteins and 4-aminobenzophenone onto cellulose substrate materials was carried out with EDC/NHS chemistry in Papers II–IV. A solution comprising 0.1 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 0.4 M N-hydroxysuccinimide (NHS) was utilized to treat TOCNF and BP-CNF surfaces. The chemicals were obtained from Sigma-Aldrich (Finland). After the EDC/NHS treatment, the surface was rinsed with aqueous buffer solution and the desired antibody was adsorbed onto the activated cellulose surface from the respective buffer solution. Unreacted NHS-esters from the surface were removed with ethanolamine (EA) (Sigma-Aldrich, Finland) treatment. Detailed descriptions of the conjugation procedure is available in Papers II and IV.

3.2 Methods

3.2.1 Cellulose model film preparation via spin-coating

Spin-coating is a relatively simple method for ultrathin film preparation where a drop of polymer solution is placed on a static surface, which is then rotated at
a certain angular velocity for a given time period (Amim et al. 2008). Excess polymer solution is removed from the substrate due to its high angular velocity and only a small layer of solution remains on its surface. Subsequently a uniform thin layer is formed as the remaining solvent evaporates, leaving only a polymer layer on top of the substrate (Kontturi et al. 2003). Figure 17 illustrates the spin-coating procedure used to prepare the ultrathin films.

![Spin-coating process](image)

**Figure 17.** Schematic representation of spin-coating an ultrathin film on top of a solid substrate.

Spin-coating was applied in this work to produce ultrathin films of TMSC (Paper I) and CNF (Papers I-IV). TMSC solution of 10 g L⁻¹ in toluene was spin-coated (Model WS-650SX-6NPP, Laurell Technologies, PA, USA) at 4000 rpm for 60 s onto silicon dioxide substrates (Kontturi et al. 2003). The TMSC surfaces were hydrolyzed to regenerated cellulose by a hydrochloric acid vapor treatment. CNF model surfaces were prepared according to the method described by Ahola et al. (2008a). The nanofibrils obtained from supernatant were spin-coated onto silicon dioxide or gold substrates with a pre-adsorbed thin layer of PEI at 3000 rpm with 90 s spinning time. The crystals were stored at ambient conditions and were allowed to stabilize in water prior to measurements.

### 3.2.2 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) allows for real time observation of adsorption events from liquid solutions onto SPR sensor surfaces (Jung et al. 1998) and has gained attention, especially in biotechnology and drug research. In this thesis, SPR was utilized to monitor TEMPO-oxidation reactions in addition to copolymer and protein adsorption and conjugation onto cellulose substrates. SPR is an optical method based on the surface plasmon resonance phenomenon, first observed by Wood (1902), but only elaborated decades later by Kretschmann and Raether (1968). An SPR sensor consists of a thin metal film e.g. gold
(~50nm), coated on a transparent optical substrate such as glass. The sensor is placed on a prism and light is passed through the prism. When the light reflects from the gold coating under total internal reflection conditions, a surface plasmon wave propagating along the analyzed solution and gold layer interface is generated as the photons from the light interact with free electrons (plasmons) of the gold film. This coupling consumes energy and a sharp attenuation of reflectivity occurs. As a result, a dip in the measured intensity of reflected light is observed. Depending on the spectrometer, monochromatic light is used when intensity versus incident angle is measured or white light can be used if intensity versus wavelength at fixed angle is measured. Adsorption can occur from liquid solution onto the metal surface. (Campbell, Kim 2007, Jung et al. 1998) The metal surface can also be coated with a nanoscale layer or coating (Jung et al. 1998). The refractive indexes (RI) of media in contact with the metal surface of the SPR sensor correlate very sensitively with the angles and wavelengths at which the surface plasmon resonance effect occurs. More information about the SPR method can be found in reviews by Pattnaik (2005) and Homola et al. (1999). Figure 18 presents a schematic illustration of the SPR technique.

\[
\Delta \text{angle} = \frac{l_d \Delta \text{angle}}{2 m(n_a-n_o)}
\]

where \( \Delta \text{angle} \) is the change in the SPR angle, \( l_d \) is a characteristic evanescent electromagnetic field decay length, estimated as 0.37 of the light wavelength (240 nm), \( m \) is a sensitivity factor for the sensor obtained after calibration of the SPR (109.94°/RIU), \( n_o \) is the refractive index of the bulk solution (1.334 RIU), and \( n_a \) is the refractive index of the adsorbed substance. A refractive index of 1.57 for proteins (Jung et al. 1998) and 1.46 for the block and random copolymers (based on POEGMA) (Lee et al. 2007) were assumed. The adsorbed amount per unit area, \( \Delta m \), was determined with equation 3.2 (Campbell, Kim 2007):
\[ \Delta m = d \times \rho \]  

(3.2)

where \( d \) is the thickness of the adsorbed layer and \( \rho \) is the packing density of the adsorbed species. The assumed packing densities were 1.3 g cm\(^{-3}\) (Campbell, Kim 2007) for proteins and 1.15 g cm\(^{-3}\) based on POEGMA (Feng et al. 2006) for the block and random copolymers.

Prior to SPR measurements, the cellulose model films were stabilized in water overnight after which they were mounted in the SPR module. The films were stabilized in continuous flow of MilliQ water or appropriate buffer solution until a stable baseline was observed. Each SPR measurement was repeated at least twice under constant flow rate of 100 \( \mu \)l min\(^{-1}\) at 20 °C. Detailed descriptions of experimental setups are available in Papers I, II and IV.

### 3.2.3 Quartz Crystal Microbalance with Dissipation

The Quartz Crystal Microbalance with Dissipation monitoring technique (QCM-D) was used in this work to monitor TEMPO-oxidation reactions as well as for copolymer and protein adsorption and conjugation. The basis of the QCM-D method is the acoustic oscillation of quartz crystal which can be measured to monitor adsorption events (Figure 19). The popularity of this method in surface science originates from its unique ability to monitor adsorption on solid surfaces in real time while simultaneously providing information about viscoelastic properties of the adsorbing layer (Rodahl et al. 1995, Höök et al. 1998). When a voltage is passed through the piezoelectric quartz crystal, it causes it to oscillate at a specific resonance frequency. When the mass of the crystal increases, the oscillating frequency decreases. The QCM-D technique is therefore susceptible to mass changes of the quartz crystal caused by either mass adsorption or coupling of liquid medium in the surface. The oscillation of the crystal is gradually decreased and stopped if the voltage is cut off, due to frictional energy losses of the adsorbing layer, measured as the dissipation of the surface.

![Figure 19. Schematic illustration of the operation of a QCM-D.](image)
In an ideal case, the adsorbed mass is proportional to the change in resonance frequency according to the Sauerbrey equation (Sauerbrey 1959, Rodahl, Kasemo 1996):

$$\Delta m = -C_{QCM-D} \frac{\Delta f}{n}$$  \hspace{1cm} (3.3)

where $C_{QCM-D}$ is 17.7 ng Hz$^{-1}$ cm$^{-2}$ for 5 MHz crystal (provided by the manufacturer), $\Delta f$ is change in frequency and $n$ is an overtone number. However, the Sauerbrey equation results in mass underestimation if (a) the adsorbed layer is not rigid or (b) it is not uniformly distributed on the crystal or (c) the adsorbed mass is substantial in relation to the mass of the crystal.

Under such conditions, the viscoelastic properties of the adsorbing layer are analyzed with the energy dissipation of the crystal as voltage is cut off and the decaying amplitude is recorded as a function of time. Rigid adsorbed layers have longer damping times compared to viscoelastic layers. The shorter damping time results from frictional losses and they are observed as higher dissipation values. The dissipation factor ($D$) of the system can be defined as:

$$D = \frac{E_{diss}}{2\pi E_{stored}}$$  \hspace{1cm} (3.4)

where $E_{diss}$ is the energy dissipated during one oscillation cycle and $E_{stored}$ is the total energy stored in the oscillating system (Höök et al. 1998).

The Voigt viscoelastic model was utilized in this work to estimate the mass of the viscoelastic adsorbed layer. The basis of this model is a purely viscous damper and a purely elastic spring connected in parallel. The calculations were performed in a Q-Tools software data analysis program (Version 2.1, Q-Sense, Västra Frölunda, Sweden). The calculation takes into account both the change in frequency and dissipation data in the iterative model (Voinova et al. 1999). The fitting parameters for this analysis are presented in more detail in Papers I and IV.

The QCM-D measurements were performed using a Q-Sense E4 instrument (Västra Frölunda, Sweden). The frequency and dissipation changes were measured at a fundamental resonance frequency of 5 MHz and its overtones. The mass sensitivity for this instrument was 0.1 Hz corresponding to 0.018 mg m$^{-2}$. Prior to measurements cellulose model films were stabilized in water overnight and subsequently mounted to the QCM-D measurement chambers. The films were stabilized in continuous flow of buffer solution until a stable baseline was observed. Every QCM-D experiment was performed at least twice under a constant flow rate of 100 µl min$^{-1}$ at 23 °C. Detailed descriptions of experimental setup, equations, and modeling parameters can be found in Papers I and IV.

**3.2.4 Atomic Force Microscopy**

Atomic Force Microscopy (AFM) was utilized in this work to study the topographical and morphological changes on modified cellulose model films. AFM
is a scanning probe microscopy technique introduced in (1986) by Binnig et al. The method has reached popularity in modern surface science since it facilitates characterization in the nanoscale without sample preparation. AFM is based on scanning a surface with sharp tip (5-10 nm radius) that is attached to a flexible cantilever. A laser beam is directed on the cantilever and is reflected to a quadrant photodiode. The cantilever with the tip scans the surface either in close or direct contact, and the interaction of the tip with the surface causes the cantilever to deflect and the reflected laser beam is detected at different parts of the quadrant photodiode with respect to the sample surface. The detected laser beam induces a voltage that is fed to a piezoelectric scanner that controls the sample movements and an image of the surface is created with imaging software. The AFM setup is presented in Figure 20.

![Figure 20. Schematic illustration of the AFM setup.](image)

AFM can be operated under three modes: contact, non-contact, and tapping mode. Only tapping mode is discussed in this work, as it is the only one applicable for soft materials, such as cellulose, to minimize tip damage to surface (Magonov et al. 1997). In tapping mode, the tip is oscillated at a constant frequency vertically while approaching contact with the surface at the lowest point. The change in amplitude of the tip is measured while the frequency is kept constant and, as a result, a three-dimensional height image is produced. Hard surfaces cause only minor changes in amplitude, but soft surfaces reduce the amplitude significantly. Detailed reports of the AFM technique can be found in literature (Rugar, Hansma 1990, Meyer 1992). The vertical resolution of AFM is \( \sim 0.1 \text{ nm} \) due to the high sensitivity of the cantilever and precision of the piezoelectric scanner. The lateral resolution of AFM, however, is limited to tip convolution (30 nm).
The AFM utilized in this work was a MultiMode 8 Scanning Probe Microscope (Bruker AXS Inc., Wisconsin, USA). Silicon cantilevers NSC15/AlBS with curvature less than 10 nm from MicroMasch (Tallinn, Estonia) were used to scan 5 × 5 μm² and 1 × 1 μm² sized images in air. At least three separate areas on each sample were imaged. Nanoscope Analysis 1.50 (Bruker Corporation) was used for analyzing and flattening was the only image-processing step applied.

3.2.5 X-Ray Photoelectron Spectroscopy

X-Ray photoelectron Spectroscopy (XPS) has gained widespread popularity in ligno-cellulosic material research since the 1990s as a highly surface-sensitive analysis method that allows for the first 10 nm of material to be probed. XPS is based on the photoelectric effect. A sample is irradiated with X-rays, which triggers the emission of photoelectrons. The kinetic energy of the photoelectron is measured by an analyzer, which is directly dependent on the binding energy of the electron to the relevant nuclei. The binding energy is element-specific, yielding XPS an element-sensitive technique. The photoelectrons can travel very short distances in matter and air due to energy lost by collisions, rendering XPS highly surface-sensitive. This, however, means that XPS must be performed in ultra-high vacuum conditions with a pressure preferably below 10⁻⁹ mbar. As the photoelectric effect was already discovered in the late 19th century (Hertz 1887) and elaborated by Einstein (1905), it is the advancement of UHV technology that has made XPS a feasible method.

The surface chemical composition of dried unmodified and modified cellulose model films in this work was examined with AXIS Ultra spectrometer by Kratos Analytical with an X-ray source of monochromatic Al Kα at 100 W. The analysis depth and area were <10 nm and ~1 mm², respectively. All spectra were collected at an electron take-off angle of 90° at several locations. Evacuation of samples in a pre-chamber overnight was performed prior to measurements. Satisfactory experimental conditions were monitored with measuring a standard filter paper reference (S&S 5893 Blue ribbon 6, ashless, 100% cellulose) in situ with each sample batch. High-resolution spectra of carbon (C 1s), oxygen (O 1s) and nitrogen (N 1s) (the latter was used as a protein and copolymer marker) was collected in addition to elemental wide-region data. Experimental parameters for chemical analysis and high-resolution carbon fits for cellulosic materials are described in detail by Johansson and Campbell (2004). The XPS technique is described in more detail by Moulder and Chastain (1992).

3.2.6 Additional methods

Wet-spinning of CNF filaments

TOCNF and BP-CNF filaments were produced by wet-spinning in Papers III and IV. Wet-spinning is a process in which filaments are produced by forcing a spinning dope, often a viscous solution or a dispersion of polymer, through a needle (Clemons 2016, Lundahl et al. 2017). After the filaments are extruded from the needle, they are submerged in an anti-solvent or coagulation bath for removal of the dope’s solvent. Residual solvent is removed upon drying of the filaments.
The wet-spinning of CNF maintains the native cellulose I structure that can contribute to better physical properties. The properties of the resultant filaments are affected by the speed of the process and needle parameters as well as by the anti-solvent used.

In this thesis, aqueous suspensions (2%) of TOCNF and BP-CNF were wet-spun to an acetone coagulation bath at a speed of 7.5 m min$^{-1}$ (10 mL min$^{-1}$) through a needle with a diameter of 1.3 mm and length of 10.5 cm. After the produced filaments were kept in the coagulation bath for ~5 min they were air-dried. Longitudinal contraction during air-drying was prevented by securing both ends of the filaments in place. The resultant dry BP-CNF and TOCNF filaments were exposed to UV light at 356 nm wavelength with 16 J cm$^{-2}$ activation energy (Uvitec CL 508L UV chamber, Cambridge, UK).

**Tensile and wet strength of CNF nanopaper and filaments**

Linear density values for wet-spun dry BP-CNF and TOCNF filaments were measured with a Vibroskop 400 apparatus (Lenzing Instruments GmbH & Co KG, Gampern, Austria). Linear density is the mass of yarn in grams per 1000 m (tex) or 10 000 m (dtex). The mechanical properties of dry CNF nanopapers and filaments were tested with an MTS 400/M vertical tester (MTS® System Corporation, Eden Prairie, USA). In the case of filaments, the measurements were also performed in wet state. A gap width of 30 mm and 2 bar pressure with a 50 N load cell were utilized under a 30 mm min$^{-1}$ testing rate. The nanopaper and filaments were conditioned at 23 °C and 50% RH before testing. All mechanical testing was carried out in the same conditions. The thickness of the nanopapers were measured utilizing an L&W Micrometer 250. The thickness of BP-CNF filaments was measured before any measurement using a manual micrometer gauge.

The wet strength of the BP-CNF and TOCNF filaments were assessed after immersion in water for at least 2 h. The thickness for wet BP-CNF filaments were measured after immersion in water immediately before tensile strength measurement. The wet BP-CNF filaments were exposed to 23 °C and 50% a couple of minutes during thickness measurement and sample mounting to MTS tester. For the non-crosslinked TOCNF filaments, the wet thickness was measured separately with an optical microscope after immersion in water for 2 h given their substantial swelling and poor wet strength. A mean thickness value for the TOCNF filaments in wet state was used in the calculation of the mechanical properties. The apparent density, specific strength, and modulus of the filaments were calculated according to Equations 3.5, 3.6 and 3.7, respectively:

$$\text{App. density (g cm}^{-3}) = \frac{\text{Linear density (g cm}^{-1})}{\text{Apparent cross-section area (cm}^{2})}$$  \hspace{1cm} (3.5)

$$\text{Specific strength (MPa cm}^{3} \text{ g}^{-1}) = \frac{\text{Tensile strength (MPa)}}{\text{Apparent density (g cm}^{-3})}$$  \hspace{1cm} (3.6)

$$\text{Specific modulus (GPa cm}^{3} \text{ g}^{-1}) = \frac{\text{Young’s modulus (GPa)}}{\text{Apparent density (g cm}^{-3})}$$  \hspace{1cm} (3.7)
Experimental

**Scanning electron microscopy (SEM)**

Scanning electron microscopy of the BP-CNFCNF and reference TOCNF filaments was performed using a JEOL field emission microscope (JSM-7500FA) at 1 kV. Prior to imaging, the filaments were sputtered with Au/Pd alloy using a glow discharge apparatus (Emitech K100X) at 30 mA for 1 min.

**Confocal Laser Scanning Microscopy (CLSM):**

Confocal laser scanning microscope images of the BP-CNFCNF filaments were acquired for Hb detection at different concentrations with FITC-labelled anti-Hb antibody. For this purpose, a Leica TCS SP2 Confocal Laser Scanning Microscope (Leica microsystems CMS GmbH, Manheim, Germany) was used operating at reflection and fluorescence image modes. The excitation wavelength was 488 nm and the detection wavelength range 500 – 540 nm. 750×750 μm² images were scanned from at least three separate areas using average mode and under constant imaging conditions (laser power 700 V). The 3D images were rendered from 60 optical sections of the CNF filaments in topography mode.

**Conductometric titration**

The increase in carboxyl content of TEMPO-oxidized wood fibers before and after aminobenzophenone functionalization was determined with conductometric titration (751 GPD Titrino, Metrohm AG, Herisau, Switzerland) according to standard SCAN-CM: 65:02. Fibers (2.5 g dry) were titrated with 0.025 ml injections of 0.1 M NaOH at 30 s intervals. The amount of weak acid i.e. carboxyl groups in the samples was calculated as described in SCAN-CM: 65:02
4. Results and Discussion

This chapter summarizes the most important findings of this doctoral thesis. More detailed results with analysis and discussion is provided in attached Papers I-IV.

4.1 Cellulose substrates

Functionalization of nanocellulose materials is essential for the feasibility of the material for use in diagnostic or biomedical applications. The hydroxyl groups of cellulose are relatively inert for robust biomolecule immobilization or require harsh reaction conditions such as non-aqueous solvents that can affect the fibrillar matrix structure. Functionalization steps, such as TEMPO-oxidation, are required for efficient and uniform attachment as discussed previously. Another prerequisite for bioactive applications is adequate water-resistivity, which unfortunately is typically worsened by the functionalization strategies required for feasible biomolecule attachment. How to overcome this paradox was one of the main targets in this work.

4.1.1 Benzophenone functionalization

The possibility to improve the wet mechanical properties of TEMPO-oxidized cellulose nanofibril materials by photo-crosslinking chemistry was investigated. Benzophenone (BP) is a photoactive group that can be utilized to covalently link molecules. Depending on the wavelength of UV light, benzophenone photolyses to a highly reactive triplet state followed by a transition to a ketyl radical via $n-\pi^*$ or $\pi-\pi^*$ transition. Through H-abstraction/recombination mechanisms with the radicalized benzophenones, they can react with hydrogens from aliphatic CH groups (Walling, Gibian 1965). Upon exposure to UV light, radicalized benzophenone groups grafted onto cellulose or cellulose nanofibrils may abstract hydrogen from either C1-carbon or C6-ketone of an adjacent cellulose chain resulting in covalent linkages between cellulose chains or fibrils.

4-aminobenzophenone was covalently crosslinked with TEMPO-oxidized CNF via EDC/NHS chemistry. A schematic representation of the process to prepare photo-crosslinked nanocellulose material with improved wet strength is presented in Figure 21. Birch wood fibers were first TEMPO-oxidized to form carboxyl groups on the native cellulose I structure. The EDC/NHS conjugation strategy was used to form stable amide bonds between the carboxyl groups on
the fiber and amino group of the benzophenone derivative. Detailed description of the crosslinking chemistry of BP-activated fibrils can be found in Paper III.

Figure 21. Schematic representation of the benzophenone functionalization of wood fibers into crosslinked fibrils (not drawn to scale).

The successful conjugation of benzophenone groups onto the TEMPO-oxidized fibers was analyzed by conductometric titration before and after benzophenone functionalization of the fibers. A 70% decrease in charged groups on fibers indicated efficient conjugation of BP groups onto the fibers. Additional information on analysis and characterization on BP conjugation is presented in Paper III.

BP-CNF filaments were prepared through wet-spinning. Ultrathin model films were also prepared from the benzophenone-functionalized fibrils and further activation of the developed material was carried out as a platform for diagnostic and biomedical purposes. The prepared BP-CNF substrates were subjected to UV light to enable photo-activated crosslinking between fibrils to achieve improved mechanical properties in wet conditions.

4.1.2 BP-functionalized CNF nanopaper

BP-CNF nanopapers were prepared from the BP-functionalized fibrils to further verify the successful conjugation of BP onto TOCNF (XPS) and to facilitate the investigation of mechanical properties of the functionalized nanopaper in dry and wet conditions. The prepared BP-CNF films were subjected to UV light to enable photo-activated crosslinking between fibrils to achieve improved mechanical properties in wet conditions. XPS was applied to investigate the surface composition of prepared CNF nanopapers. BP-functionalized samples exhibited typical characteristics to cellulose but a distinct nitrogen peak (2%) was observed, additionally validating the successful formation of amide bonds between TOCNF and aminobenzophenone (Paper III).
The mechanical testing of the BP-CNF and TOCNF nanopaper prepared by pressure filtration revealed that the apparent density of the functionalized nanopapers decreased significantly compared to that of the unmodified TOCNF. The apparent thickness of the BP-CNF nanopaper was considerably higher than that of the TOCNF nanopaper. These observations suggest that the BP functionalization introduced hydrophobicity to the fibrils, making the fibrils more rigid in aqueous environment and resulting in films with higher thickness and less dense structure. Tensile testing (Table 1) of the dry BP-CNF nanopapers indicated that the BP functionalization increased specific strength (eq. 3.6) of the nanopaper from 109 ± 10 MPa cm³ g⁻¹ (TOCNF) to 114 ± 23 MPa cm³ g⁻¹ (BP-CNF without UV). UV activation of the film further improved the strength to 138 ± 10 MPa cm³ g⁻¹. Sonication tests of BP-CNF nanopapers indicated that the wet strength of the nanopaper was significantly improved by BP functionalization and UV curing since the UV-treated BP-CNF paper was the only one to remain unchanged after 24 h water immersion and 2 min sonication (Paper III).

Table 1. Mechanical properties of BP-CNF with and without UV activation and TOCNF (included as a reference) nanopapers.

<table>
<thead>
<tr>
<th></th>
<th>TOCNF</th>
<th>BP-CNF without UV</th>
<th>BP-CNF with UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent density (g cm⁻³)</td>
<td>1.0 ± 0.38</td>
<td>0.66 ± 0.32</td>
<td>0.70 ± 0.44</td>
</tr>
<tr>
<td>Apparent thickness (μm)</td>
<td>54 ± 2</td>
<td>84 ± 4</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>Specific tensile strength (MPa cm³ g⁻¹)</td>
<td>109 ± 10</td>
<td>114 ± 23</td>
<td>138 ± 10</td>
</tr>
<tr>
<td>Elongation (%)</td>
<td>3.3 ± 0.6</td>
<td>2.7 ± 1.2</td>
<td>2.0 ± 0.5</td>
</tr>
</tbody>
</table>

4.1.3 Wet-spun BP-CNF filaments

BP-CNF filaments were successfully prepared by wet-spinning with acetone coagulation. Fibrous filament structures were obtained from the BP-CNF suspension due to immediate dehydration in acetone with typical diameters of 133 ± 10 μm. SEM images were taken from the resultant BP-CNF and reference wet-spun TOCNF filaments to analyze the topology of the surfaces (Figure 22). Both the BP-CNF and TOCNF filaments exhibited rough surfaces but the BP-CNF filaments showed more creases along the filament’s axis in comparison to the reference TOCNF filaments (Figure 22a and b). The SEM images (Figure 22a and b) also revealed that the spun BP-CNF and TOCNF filaments maintained the cylindrical shape of the spinning needle. These observations fit quite well to the reported diverse morphologies of wet-sun cellulose I filaments in the few studies available on the subject (Iwamoto et al. 2011, Walther et al. 2011, Lundahl et al. 2016, Clemons 2016, Lundahl et al. 2017).
The mechanical properties of the BP-CNF and reference TOCNF filaments were measured with Vibroskop 400 and MTS 400 apparatuses and the results are presented in Table 2. Titer (linear mass density, expressed in grams per 1000 meters, tex, or per 10,000 meters, dtex) of the BP-CNF filaments was found to be substantially higher than for unmodified TOCNF filaments being $122 \pm 3.3$ and $98.1 \pm 16$ dtex, respectively. The substantially higher titer of the BP-CNF filaments compared to the reference filaments was the result of poorer dispersion of the BP-CNF fibrils in water than of the reference TOCNF fibrils, leading to looser spun filaments from the BP-CNF fibrils. This effect could also be seen in the lower density of the BP-CNF filaments compared to the reference TOCNF filaments (Table 2).

In addition, the UV treatment of the filaments induce the benzophenone groups to form covalent bonds between the fibrils, increasing the strength of the material. The conjugation efficiency of the BP-CNF fibers was determined to be above 70% with conductometric titration (Paper III). The specific strength values (eq. 3.6) of the BP-CNF and TOCNF filaments are quite similar (Table 2) as was observed for the nanopapers. The strain at break (from TOCNF 2.8% to BP-CNF 1.3%) of the filaments decreased somewhat upon benzophenone modification and UV treatment, which is logical since the covalent bonds between fibrils make the material harder but more brittle. The stiffness increase of the filaments was also confirmed by the decrease in the calculated specific modulus (eq. 3.7) (Table 2).

When the wet tensile strength values of the TOCNF and BP-CNF filaments were compared it was evident that the BP treatment remarkably increased the wet strength of the filaments, from $0.44 \pm 0.04$ MPa to $103 \pm 38$ MPa, respectively (Table 2), indicating an 230-fold increase in wet strength upon BP functionalization and UV crosslinking. The minimal drop in tensile strength of the BP-CNF filaments upon immersion in water (from 127 to 103 MPa) is remarkable and demonstrates the effectiveness of the BP crosslinking in producing robust water-resistant filaments. The results indicate residual dry strength of ~81%, a remarkable value if compared to recent efforts in this field (Yao et al. 2017, Mertaniemi et al. 2016) and to the performance of highly sized cellulose fibers in paper sheets (Dunlop-Jones 1996). These observations for functional-
ized filaments as well as for nanopaper demonstrate that the benzophenone activation and subsequent UV treatment of wet-spun BP-CNF filaments can be applied to substantially increase the wet strength of TEMPO-oxidized cellulose nanofibril filaments a precursor for further developments. The next material requirement to be addressed for feasible utilization of nanocellulose for bioactive materials was the issue of non-specific adsorption of proteins onto highly reactive TOCNF surfaces.

Table 2. Mechanical properties of wet-spun BP-CNF and TOCNF filaments measured in dry and wet states. Apparent density values were calculated based on linear mass density and apparent thickness (Eq. 3.5). The specific strength and modulus were obtained using Equations 3.6 and 3.7.

<table>
<thead>
<tr>
<th></th>
<th>TOCNF (dry)</th>
<th>TOCNF (wet)</th>
<th>BP-CNF (dry)</th>
<th>BP-CNF (wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear density</td>
<td>98.1 ± 16</td>
<td>N/A</td>
<td>122 ± 3.3</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Apparent thickness</td>
<td>90 ± 4.7</td>
<td>367 ± 35</td>
<td>133 ± 10</td>
</tr>
<tr>
<td></td>
<td>Apparent density</td>
<td>1.22 ± 0.14</td>
<td>0.07 ± 0.01</td>
<td>0.70 ± 0.10</td>
</tr>
<tr>
<td>Tenacity (cN/tex)</td>
<td>15.3 ± 1.7</td>
<td>0.47 ± 0.04</td>
<td>14.2 ± 2.2</td>
<td>10.8 ± 3.0</td>
</tr>
<tr>
<td>Strain at break (%)</td>
<td>2.8 ± 0.5</td>
<td>0.07 ± 0.03</td>
<td>1.3 ± 0.3</td>
<td>3.2 ± 1.8</td>
</tr>
<tr>
<td>Young’s modulus (GPa)</td>
<td>16.0 ± 2.3</td>
<td>N/A</td>
<td>11.6 ± 1.7</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>Specific modulus</td>
<td>13.1 ± 1.7</td>
<td>N/A</td>
<td>17.0 ± 4.1</td>
<td>9.8 ± 1.5</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>237 ± 33</td>
<td>0.44 ± 0.04</td>
<td>127 ± 31</td>
<td>103 ± 38</td>
</tr>
<tr>
<td>Specific strength</td>
<td>195 ± 22</td>
<td>6.1 ± 1.6</td>
<td>181 ± 28</td>
<td>138 ± 38</td>
</tr>
</tbody>
</table>

4.2 Passivation of nanocellulose

Sufficient surface passivation of the cellulosic materials to be utilized in various biomedical applications is paramount. Common blocking agents to reduce non-specific adsorption include natural proteins, such as bovine serum albumin (BSA), which adsorb on the surface saturating it, reducing subsequent non-specific binding. Although very economical, the efficiency of the BSA blocking layer needs to be optimized for hydrophilic and hydrophobic surfaces individually as well as for each protein due to complex adsorption behavior of BSA. (Sweryda-Krawiec et al. 2004, Jeyachandran et al. 2010) As also discussed previously, prominent methods to produce low-biofouling surfaces include polymer coatings consisting of e.g. polyvinyl alcohol (Amanda, Mallapragada 2001) or PEG
Results and Discussion

(Scott, Murad 1998, Huang et al. 2001, Pasche et al. 2003, VandeVondele et al. 2003, Maddikeri et al. 2008, Deng et al. 2014, Islam et al. 2014a). The challenge remains that PEG molecules do not possess strong affinity with cellulosic materials. To this end the adsorption mechanisms of block and random copolymers consisting of OEGMA (oligo(ethylene glycol)methyl ether methacrylate) monomers and DMAEMA (2-(dimethylamino)ethyl methacrylate) monomers on different cellulosic surfaces was investigated. More details about the experimental setup and results are provided in Papers I and II.

4.2.1 Block copolymer adsorption and interfacial water expulsion

Recently, polymers containing surface-passivating and surface-binding domains have been found to be very effective in producing antifouling surfaces towards proteins (VandeVondele et al. 2003, Zhang et al. 2012b, Zhang et al. 2013b, Zhang et al. 2013a). Therefore, adsorption of engineered block and random copolymers containing quaternized DMAEMA monomers and highly hydrophilic OEGMA monomers was studied on differently charged cellulose substrates. The nine block copolymers and four random copolymers are presented in Table 3. The characteristics of the utilized copolymers are provided in detail in Paper I.

Table 3. Properties of utilized PDMAEMA-POEGMA block (-b-) and random (-rnd-) copolymers with sample codes.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>DMAEMA</th>
<th>OEGMA</th>
<th>Mn (Da)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>D33-b-EGMA</td>
<td>33</td>
<td>52</td>
<td>19000</td>
<td>1.21</td>
</tr>
<tr>
<td>D33-b-EGMA</td>
<td>33</td>
<td>94</td>
<td>36600</td>
<td>1.13</td>
</tr>
<tr>
<td>D33-b-EGMA</td>
<td>33</td>
<td>137</td>
<td>58400</td>
<td>1.29</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>58</td>
<td>10</td>
<td>17100</td>
<td>1.20</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>58</td>
<td>62</td>
<td>35900</td>
<td>1.25</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>58</td>
<td>118</td>
<td>49200</td>
<td>1.23</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>74</td>
<td>17</td>
<td>17100</td>
<td>1.17</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>74</td>
<td>54</td>
<td>31400</td>
<td>1.27</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>74</td>
<td>118</td>
<td>51300</td>
<td>1.29</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>46</td>
<td>139</td>
<td>73000</td>
<td>1.28</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>86</td>
<td>67</td>
<td>45300</td>
<td>1.33</td>
</tr>
<tr>
<td>D58-b-EGMA</td>
<td>159</td>
<td>23</td>
<td>35800</td>
<td>1.29</td>
</tr>
<tr>
<td>D222-rnd-EGMA</td>
<td>222</td>
<td>7</td>
<td>38000</td>
<td>1.32</td>
</tr>
</tbody>
</table>

Block copolymers with varying segment sizes of PDMAEMA and POEGMA were adsorbed on the cellulosic substrates with QCM-D monitoring. The cellulosic substrates utilized were low-charged regenerated amorphous cellulose, medium-charged cellulose nanofibrils (CNF) and highly charged TEMPO-oxidized
Results and Discussion

Figure 23 presents QCM-D frequency and dissipation shifts upon adsorption of block copolymers D$_{33}$-b-EGMA$_{137}$ and D$_{74}$-b-EGMA$_{17}$ onto differently charged cellulosic model films.

The tertiary amine groups of PDMAEMA blocks were quaternized (permanent cationic charges) by methylation meaning that the positive charge of the block copolymers was not pH-dependent. The adsorption of PDMAEMA-b-POEGMA copolymers on cellulosic substrates exhibited surface-charge dependent behavior. Minuscule or negligible adsorption of block copolymers on regenerated cellulose (Figure 23a and c) was observed independent of block sizes, this is most likely due to the lack of affinity between PEG molecules and cellulose (Filpponen et al. 2012), in addition to the absence of electrostatic interactions between the cationic block and the low-charged cellulose surface.

The adsorption increased somewhat onto medium-charged CNF (Figure 23a and c). The wood fibers used to produce CNF by mechanical disintegration were slightly anionic due to incomplete removal of hemicelluloses during delignification (Sjöström 1989), resulting in moderate negative charges from residual carboxyl groups (pK$_a$ ~4.8 (Wågberg et al. 2008)) at the adsorption pH. Therefore,
the slightly increased adsorption of the block copolymers onto CNF compared to that on regenerated cellulose is due to the increased electrostatic interaction between the moderately anionic CNF and cationic segment of the block copolymers. However, D_{33}-b-EGMA_{37} adsorbed more extensively on CNF than D_{74}-b-EGMA_{17}, possibly due to the fact that the mass of the polymer increased as the size of the POEGMA segment was increased.

The adsorption of the block copolymers was substantially higher with very fast adsorption kinetics on TOCNF than on regenerated cellulose or CNF (Figure 23a and c), further verifying electrostatic interactions as the underlying adsorption mechanism. The carboxylic acid groups on the TOCNF surface introduced by TEMPO-oxidation were protonated at the adsorption pH 6.8 as the pKₐ value of carboxylic acids is in the range of 3.0–5.5 (Sjöström 1989). This indicates that the substantial adsorption of the block copolymers on TOCNF was driven by electrostatic interactions between the cationic PDMAEMA segments of the block copolymer and the anionic TOCNF. This is similar as previously reported for PDMAEMA-containing block copolymer adsorption on anionic mica, silicon dioxide, and cellulose surfaces. However, as these studies involved DMAEMA co-polymerized with hydrophobic blocks, other effects can also be present (Nurmi et al. 2010, Wang et al. 2011).

The dissipation values upon block copolymer adsorption onto cellulosic surfaces was also measured simultaneously with frequency shifts in QCM-D (Figure 23b and d). The cellulose model films were fully hydrated before commencing copolymer adsorption. Upon block copolymer adsorption, significant reduction in dissipation values was observed (Figure 23b and d). This is most likely due to vast amount of water expulsion from TOCNF due to charge neutralization. The large water removal also caused peculiar frequency shifts during block copolymer adsorption: in some cases, substantial positive frequency changes were observed. As QCM-D is sensitive to the mass increase either from adsorbate or coupled water, adsorption of the block copolymers on TOCNF was further studied with SPR. SPR measures the adsorbed layer thickness and mass based on the layer refractive index, which is not largely affected by changes in water coupling. Corresponding QCM-D and SPR sensograms of block copolymer adsorption onto TOCNF are presented in Figure 24.

SPR measurements confirmed substantial adsorption of all the block copolymers onto TOCNF. Therefore, it can be concluded that the unexpected positive QCM-D frequency shifts were indeed caused by charge neutralization induced by expulsion of water from the interface. The adsorption of PDMAEMA-b-POEGMA copolymers onto TOCNF (SPR, Figure 24b) indicated behavior typical for molecules adsorbing by electrostatic interactions (Hoogeveen et al. 1996). Adsorption plateau was reached very rapidly, within few minutes and rinsing did not remove the copolymers to a significant extent. Analysis of the D_{33}-EGMA_{52} and D_{74}-EGMA_{54} indicate that adsorption on TOCNF increased as the size of the PDMAEMA increased. This is due to more pronounced electrostatic interactions between anionic TOCNF and the larger cationic block as well as increased entropy by adsorbing polymer chains replacing counterions. Data comparison for D_{74}-EGMA_{17} and D_{74}-EGMA_{118} adsorption onto TOCNF.
suggests that adsorption was also increased by a larger hydrophilic block. This correlates well with the interpretation of QCM-D results for block copolymer adsorption onto CNF, where the adsorbed mass increased possibly due to the large, dangling POEGMA blocks that bind water extensively (Larsson et al. 2013).

![Figure 24](image.png)

**Figure 24.** QCM-D frequency shifts (a) and SPR angle changes (b) upon block copolymer adsorption onto highly-charged TOCNF. Block copolymer introduction to the SPR module is indicated by vertical arrows. Rinsing at given times with respective background solution (PBS, pH 6.8) is indicated with droplet symbols.

### 4.2.2 Adsorption of block copolymers vs. random copolymers

The effect of PDMAEMA-POEGMA copolymer structure on adsorption was investigated. Detailed descriptions about the copolymers can be found in Paper I. Quaternized PDMAEMA-<i>rnd</i>-POEGMA copolymers with specific molecular ratios, $D_{46}$-EGMA$_{139}$ (10-90 mol-%), $D_{60}$-EGMA$_{67}$ (30-70 mol-%), $D_{159}$EGMA$_{23}$ (70-30 mol-%), and $D_{222}$EGMA$_{7}$ (92-8 mol-%) in MilliQ water were adsorbed on TOCNF monitored by SPR (Figure 25). From Figure 25 it is evident that the adsorption isotherms differed substantially from typical Langmuir isotherms. Initially the random copolymers adsorbed rapidly on the surface after which the adsorption decelerated to a more gradual rate. The fast initial adsorption was most likely the result of electrostatic interactions between the cationic groups in DMAEMA with the anionic TOCNF surface. The more gradual adsorption after the fast initial adsorption might be the result of multilayer formation, polymer exchange between copolymers of different size (given the polydispersity), polymer re-conformation on the surface, or slow counterion release from the quaternized copolymer layer. Generally, the adsorption increased with the ratio of OEGMA units in the copolymer, due to the higher molecular mass of the OEGMA monomer compared to that of the DMAEMA monomer (Larsson et al. 2013).
Results and Discussion

The adsorption kinetics (Figure 25) and adsorbed amounts (Paper I, SI) of quaternized PDMAEMA-rnd-POEGMA on TOCNF differed somewhat from the respective block copolymers. For the block copolymers adsorption was fast and a plateau was reached in few minutes (Figure 24b). In contrast, for the random copolymers, fast initial adsorption was followed by a more gradual rate (Figure 25). The cationic segments of the block copolymers likely adsorbed tightly in flat conformation on TOCNF while the POEGMA segments extended into the solution. In the case of the random copolymers, the DMAEMA monomers or segments adsorbed tightly on the TOCNF and the OEGMA segments formed loops and tails extending to the solution. The proposed adsorption behavior of block and random PDMAEMA-POEGMA copolymers is schematically represented in Figure 26. However, in the absence of specific conformational or structural assessment of copolymer/TOCNF conjugates, other conformations due to, e.g., intermolecular interactions between individual or aggregated copolymers are also possible.
If the calculated adsorbed amounts for random D$_{33}$-EGMA$_{337}$ (6.1 ± 0.6 mg m$^{-2}$) and block D$_{46}$-EGMA$_{39}$ (20.1 ± 1.9 mg m$^{-2}$) copolymers containing similar molecular ratios of DMAEMA and OEGMA and similar molecular mass (10-90 mol-% and 7-93 mol-%, 73 kDa and 70 kDa, respectively) are compared (equations 3.1 and 3.2), it is evident that adsorption was substantially higher for random copolymers on TOCNF. Generally, the molar masses were higher for the tested random copolymers than for the block copolymers (Paper I) that can to some extent, account for the higher observed adsorption on TOCNF. However, the behavior and kinetics of adsorption of random copolymers still require further studies.

4.2.3 Controlling protein affinity

Non-specific protein adsorption is a challenge in developing successful and accurate biosensors and in various biomedical applications, such as implants. The design of many bioassays is based on the enzyme-linked immunosorbent assay (ELISA) principle, which utilizes specific antibody-antigen interactions. Therefore, it is of critical importance to minimize non-specific adsorption in order to achieve reliable and accurate results (Kenna et al. 1985). In implantable devices, it is also important to produce anti-fouling surfaces to reduce rejection and inflammation (Vaddiraju et al. 2010). Recently, highly effective surface passivation has been achieved with polymers with surface-binding and surface passivating domains. PEG molecules have been previously proven very effective in reducing non-specific adsorption. As the adsorption mechanism of block and random copolymers consisting of DMAEMA and OEGMA was determined to be mainly driven by electrostatic interactions between the cationic segments of PDMAEMA and anionic TOCNF, we explored these copolymers for surface passivation of TOCNF towards antibodies (Figure 27). Six block and two random copolymers were studied to investigate the surface passivating properties of the adsorbed copolymer layers and to shed light on the copolymer structure on surface passivation.
Results and Discussion

Firstly, the adsorption of human IgG (hIgG) on TOCNF was found to be significant (Figure 28a), indicating that accurate bioassay development on this material can present challenges in the absence of an efficient blocking agent. The isoelectric point of hIgG is reported to be between 6.5–9.5 (Chiodi et al. 1985). The substantial adsorption of hIgG on TOCNF at pH 7.4 observed in this work was most likely due to the maximal adsorption observed for proteins under isoelectric conditions, as at close to neutral net charge proteins can become more compact and pack in a denser layer on the surface (Malmsten 2000, Norde 2008). Detailed analysis of the hIgG adsorption can be found in Paper II. The ability of PDMAEMA-POEGMA block and random copolymers to block non-specific hIgG adsorption onto TOCNF was investigated next (Figure 28). Figure 28 presents the typical SPR sensograms for block (Figure 28b) and random copolymer (Figure 28c) passivation of TOCNF towards hIgG with reference measurement of non-specific hIgG adsorption on TOCNF (Figure 28a) and AFM images of dried TOCNF without and with adsorbed D$_{37}$-b-EGMA$_{137}$ (Figure 28d and f) and subsequent hIgG adsorption (Figure 28e and g).
The block and random copolymers resulted in non-specific hIgG adsorption reductions ranging between 85 – 100% and 84 – 100%, respectively. The increased amount of OEGMA in the copolymer had a favorable effect on the blocking efficiency (Figure 28), which is to be expected as PEG molecules exhibit anti-fouling properties due to steric stabilization and excluded-volume effects (Huang et al. 2001, Pasche et al. 2003, VandeVondele et al. 2003, Maddikeri et al. 2008, Scott, Murad 1998, Kenausis et al. 2000). On the other hand, with the increasing DMAEMA monomer density the blocking effect was decreased (Figure 28). As discussed previously (Paper I), in the case of the block copolymers, the PDMAEMA segments likely adsorbed tightly and in flat conformation on TOCNF, while the POEGMA block extended into solution as “tails”. In the theoretical scaling laws by Marques and Joanny (1989), block copolymers of similar segment sizes with a block with strong affinity for the surface (A) and with a block strongly repelled by the surface (B), are discussed. If the block copolymer...
Results and Discussion

is adsorbed on the surface in non-selective solvent block B forms a layer of tails extending to the solution while block A forms a swollen adsorbed layer on top of the surface. In this case, PDMAEMA is representative of block A and POEGMA of block B. If the size of block A is increased, the amount of A-B junction points is increased, leading to increased density of extending block B tails. Therefore, it can be concluded that with shorter PDMAEMA segment length, the extending POEGMA blocks became more densely packed on the surface, resulting in better protein resistance. Similarly, in the case of random copolymers, when amount of surface binding DMAEMA in the copolymer is low, the density of extending OEGMA loops and tails is increased, leading to more pronounced antifouling effects.

Random copolymer D_{16-rnd}-EGMA_{139} and block copolymer D_{33-b}-EGMA_{137} with similar molar ratios (10 % DMAEMA and 90 % OEGMA), completely rejected hIgG adsorption on TOCNF. For molar ratios of 70 % DMAEMA and 30 % OEGMA, i.e., random copolymer D_{199-rnd}-EGMA_{23} and block copolymer D_{58-b}-EGMA_{10}, minor difference (84 and 88%, respectively) in blocking efficiency was observed. Overall, it can be concluded that copolymer structure did not seem to have a significant effect on the blocking ability. Indeed, it has been found that the ability of grafted polymers to resist protein adsorption depends mainly on the dense spatial packing of side chains rather than on the backbone length or grafting ratio of the polymer (Kenausis et al. 2000). In this work both block and random copolymer were able to produce adequately densely packed layers of OEGMA extending to the solution to achieve anti-fouling TOCNF surfaces.

AFM images of TOCNF before and after hIgG adsorption (Figure 28d and e) reveal that upon hIgG adsorption the fibril features of TOCNF become less obvious. The decreased RMS roughness value of reference TOCNF surface (4.4 nm) upon hIgG adsorption (3.0 nm) indicates a smoother surface, likely due to void filling by the adsorbed protein. This is in accordance with previous SPR results indicating substantial non-specific adsorption of hIgG onto TOCNF (Figure 28a). In the case of D_{33-b}-EGMA_{137}-treated TOCNF before and after hIgG adsorption (Figure 28f and g), no changes were observed in RMS roughness (4.2 nm) further confirming the result from SPR that D_{33-b}-EGMA_{137} blocking of TOCNF resulted in total hIgG rejection.

The performance of conventional blocking agents applied in biochemistry was also tested on TOCNF and analyzed in relation to the copolymers. Typical blocking agents SuperBlock® (commercial blocking agent) and bovine serum albumin (BSA) were tested against the copolymers in their ability to reduce hIgG adsorption onto TOCNF. Additionally, the blocking efficiency of methoxypolyethylene glycol amine (PEG-amine) was studied, as it exhibited similar structural components as the copolymers but is significantly smaller in size. Further information about blocking hIgG adsorption by these substances is provided in Paper II. Figure 29 presents the ability of all tested blocking agents tested in this work to reduce non-specific hIgG adsorption onto TOCNF.
Results and Discussion

Figure 29. Adsorbed amount of human IgG (equations 3.1 and 3.2) on TOCNF after passivation with different blocking agents (blue) and included as a reference non-specific adsorption of human IgG on unmodified TOCNF (red). Corresponding blocking efficiencies i.e. reduction in human IgG adsorption are indicated as percentages.

Interestingly, typically used blocking agents, SuperBlock® and BSA, were found to be unsuitable as blocking agents for hIgG on TOCNF, since they were able to reduce non-specific adsorption by only 15 and 23%, respectively. PEG-amine was observed to be more effective in non-specific hIgG adsorption prevention reducing 75% of adsorption, but it still fell significantly short compared to the values obtained with block and random copolymers of PDMAEMA and POEGMA (84 – 100%). The PEG-amine likely adsorbed on TOCNF through electrostatic interactions between the anionic surface and positive amine groups at pH 7.4. This indicates that the unbranched and short PEG chains were not able to form thick and hydrated enough layer on top of the TOCNF to obtain very high or total hIgG rejection as opposed to the copolymers. The remarkable blocking efficiencies achieved with PDMAEMA and POEGMA block and random copolymers in this work are similar or better than those reported in the literature for IgG (Zhang et al. 2012b, Deng et al. 2014). The presented methodology utilizing block and random copolymers with cationic segments as anchoring agents for protein-resistant segments is a fast, highly effective, and simple way to gain antifouling TOCNF surfaces for hIgG. This is a major precursor in designing accurate and efficient biosensors or bioactive materials on TOCNF and presents opportunities for high-performance and reliable biosensor development.
4.3 Preparation of bioactive multi-functional nanocellulose

4.3.1 Biointerface preparation on BP-CNF

Antibody biointerface preparation through conjugation with EDC/NHS chemistry has been previously demonstrated as a simple and effective method on a number of substrates, including carboxymethyl dextran (Löfås, Johnsson 1990, Löfås et al. 1995), PEI/CMC (Carrigan et al. 2005b, Carrigan et al. 2005a), and PEI/CMC/PEG hydrogels (Carrigan, Tabrizian 2005), and various cellulosic materials, such as CMC-modified CNF (Orelma et al. 2012c, Orelma et al. 2012b) and TOCNF surfaces (Orelma et al. 2012a). In this doctoral study a method to produce water-resistant nanocellulose with photo-activation of grafted benzophenone was introduced. In order to study the potential of this developed bifunctional material, anti-human hemoglobin (anti-Hb) molecules were attached via EDC/NHS coupling. A schematic representation of the process is given in Figure 30.

Figure 30. Schematic illustration of EDC/NHS conjugation of anti-Hb onto benzophenone functionalized TEMPO-oxidized cellulose nanofibrils (BP-CNF) for hemoglobin (Hb) detection.

First, anti-Hb was conjugated via EDC/NHS activation onto TOCNF and monitored with either SPR or QCM-D. Detection of Hb by the biointerface was then tested. The corresponding SPR and QCM-D sensograms upon EDC/NHS activation and anti-Hb conjugation are presented in Figure 31, along with reference measurement for non-activated BP-CNF surfaces.
Results and Discussion

Figure 31. SPR angle changes (a) and QCM-D frequency shifts (b) upon anti-Hb adsorption (0.1 mg ml⁻¹, 10 mM acetate buffer, pH 5) onto BP-CNF model film with (red) and without (black) EDC/NHS activation and ethanol amine (EA) treatment. Included also Hb (red, solid symbol) and HSA (red, open symbol) adsorption (0.1 mg ml⁻¹, 10 mM PB, pH 7.4) onto anti-Hb-conjugated BP-CNF i.e. anti-Hb biointerface monitored with SPR (c) or QCM-D (d). Arrow symbols indicate substance injection into respective measurement module and droplet symbols indicate rinsing with respective buffer solution.

From Figure 31 it is obvious, that substantial amount of anti-Hb was conjugated onto the BP-CNF. The remaining carboxyl groups on BP-CNF, after BP functionalization, facilitated the successful conjugation of anti-Hb on TOCNF with EDC/NHS. Upon ethanolamine treatment, some of the attached anti-Hb was removed from the surface. However, in the case of the reference measurements, with no EDC/NHS activation, almost all anti-Hb was removed from the BP-CNF (Figure 31a and b). Ethanolamine was introduced into the system to remove unreacted NHS ester from the surface to corresponding ethylamides to prevent interference in further process steps. The removal of some antibodies upon EA treatment has been reported previously and has been attributed to charge repulsion between physically adsorbed antibodies and anionic TOCNF. The pH of EA solution is typically 8.5 and the net charge of the antibodies was shifted to more negative (pI 6.5 – 9.5)(Chiodi et al. 1985), resulting in charge repulsion between anionic BP-CNF and desorption of physically adsorbed anti-Hb.
Results and Discussion

After ethanolamine treatment, the conjugated and adsorbed amounts of anti-Hb on BP-CNF were determined to be 2.4 ± 0.1 and 0.4 ± 0.01 mg m⁻², respectively (equations 3.1 and 3.2) in SPR and 5.0 ± 2.1 and 1.9 ± 0.8 mg m⁻², respectively (Voigt viscoelastic model) in QCM-D. These values were somewhat lower than previously reported for ECD/NHS conjugation of anti-Hb onto CMC-modified cellulose in SPR and QCM-D, 4.6 and 5.5 mg m⁻² (Orelma et al. 2012c), respectively, as was expected since some of the carboxylic groups of TOCNF were already utilized for BP functionalization. However, the results still indicate that BP functionalization did not significantly impair the potential of the material for conjugation. Other reported conjugated amounts of proteins with EDC/NHS chemistry onto CMC/PEI hydrogels or carboxymethyl dextran surfaces indicate values up to 7.8 (Carrigan et al. 2005b) and 15 (Löfås, Johnsson 1990) mg m⁻², respectively. While values of attached anti-Hb obtained in this work were lower, the potential of BP-CNF as a platform for protein conjugation was proven and evidence of the bifunctionality of the material was provided. Based on these results and the mechanical properties discussed the previously BP-CNF is demonstrated to be a bifunctional material platform with adequate potential for protein conjugation and sufficient wet mechanical strength.

The selectivity of the prepared anti-Hb biointerfaces towards Hb was tested by adsorbing Hb and HSA on the surface with SPR and QCM-D monitoring (Figure 31 c and d). HSA was chosen as a model protein with no affinity for anti-Hb to demonstrate the selectivity of the system. From Figure 31 it is evident that the anti-Hb biointerface was highly specific for Hb, with adsorbed amounts of 1.7 ± 0.12 and 2.0 ± 1.0 mg m⁻², while very low adsorption levels, 0.11 ± 0.01 and 0.3 ± 0.1 mg m⁻², for HSA were observed in SPR and QCM-D, respectively. The amount of bound Hb was even higher than previously reported for CMC-modified cellulose (Orelma et al. 2012c), indicating remarkable sensitivity for the developed bifunctional material platform based on BP-CNF.

4.3.2 Copolymer effect on antibody binding

The block and random copolymers comprising PDMAEMA and POEGMA were previously determined to be excellent in preventing non-specific adsorption of human IgG hemoglobin onto TOCNF. The effect of the copolymer on the functionality on antibody biointerface prepared through EDC/NHS activation on TOCNF was also investigated. While offering remarkable antifouling properties to the TOCNF surface it is essential in some applications, such as biosensors, that the blocking does not affect the specific antigen-antibody interaction crucial for bioassay development. XPS measurements were performed to assess the surface chemical composition upon different stages of biointerface preparation with block copolymer D₃₃-b-EGMAₙ₇₋, which was determined to totally reject hIgG. Table 4 presents the XPS atomic concentrations for dried samples at different stages of biointerface preparation on TOCNF.
Table 4. XPS atomic concentrations (%) for different stages of anti-human IgG (anti-hIgG) biointerface preparation on TOCNF with EDC/NHS conjugation.

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<thead>
<tr>
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<th>Atomic concentration (%)</th>
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<tr>
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<td>C 1s</td>
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<tr>
<td>TOCNF (ref)</td>
<td>43.7 ± 0.9</td>
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<tr>
<td>Anti-hIgG biointerface</td>
<td>46.7 ± 2.1</td>
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<tr>
<td>Anti-hIgG biointerface with D_{33^-b-EGMA}_{137}</td>
<td>57.4 ± 0.8</td>
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<tr>
<td>Anti-hIgG biointerface with D_{33^-b-EGMA}_{137} and hIgG</td>
<td>61.7 ± 0.9</td>
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From the XPS data, it can be determined that upon anti-hIgG conjugation with EDC/NHS chemistry the amount of nitrogen on TOCNF increased clearly from 0.90 to 1.95%. The N signal remained similar (1.95 vs. 1.64%) upon block copolymer D_{33^-b-EGMA}_{137} adsorption onto the biointerface but the peak was divided into two distinct spectral contributions (Paper II), likely originating from amide and quaternary ammonium groups. Negligible changes in nitrogen content of the surface (from 1.64 to 1.77%) was observed upon hIgG adsorption onto the biointerface, this is likely the result of somewhat low adsorption of hIgG compared to the conjugated amount of anti-hIgG and adsorbed amount of block copolymer D_{33^-b-EGMA}_{137} on the surface.

Anti-human IgG biointerface preparation onto TOCNF with D_{33^-b-EGMA}_{137} was also monitored with SPR (Figure 32a and c). As a reference, biointerface preparation without block copolymer is also presented (Figure 32b and c).
Figure 32. SPR sensograms upon anti-hIgG biointerface preparation on TOCNF with EDC/NHS chemistry and ethanolamine (EA) treatment followed by hIgG detection with (a) and without (b) surface passivation by D$_{33}$-$b$-EGMA$_{137}$. Schematic representation of the biointerface response to hIgG with (c) and without (d) sufficient surface passivation.

From Figure 32 it is evident, that adequate surface passivation played an important role in accurate and reliable bioassay development based on TOCNF materials. In the SPR signal, a substantial difference in response upon hIgG adsorption to the biointerface with (Figure 32a) and without (Figure 32b) D$_{33}$-$b$-EGMA$_{137}$ blocking was observed. The adsorbed amount of hIgG was determined to be 0.90 ± 0.09 and 7.8 ± 0.2 mg m$^{-2}$ for the biointerfaces with and without passivation, respectively. Remarkably, the hIgG detection without blocking was nine-fold compared to the actual detection response when block copolymer passivation was applied. In addition to the specific antigen-antibody interactions of the biointerface with hIgG, the substantial false hIgG response of the biointerface was likely the result of incomplete EDC/NHS activation of the surface allowing for electrostatic interactions with remaining carboxylic groups on the surface as well as to the decreased solvency of hIgG near its isoelectric point (Chiodi et al. 1985). The results indicated that unless careful steps are taken to ensure sufficient surface passivation on TOCNF, biosensor accuracy could be seriously affected by significant non-specific adsorption. They also demonstrate that as the block and random copolymer of POEGMA and PDMAEMA provide much-needed passivation for the TOCNF surface to remove false signals they do not compromise the functionality or reduce the performance of the antibody biointerface.
4.3.3 Biointerface response to concentration and regeneration

In order to tune the properties of the prepared biointerfaces and to allow for multiple test cycles, the properties of the biointerfaces were probed against concentration and regeneration steps. The effect of target molecule concentration on the binding of the anti-Hb biointerface prepared on BP-CNF model films was studied with both SPR and QCM-D. The anti-Hb biointerface on BP-CNF was found to exhibit concentration-dependent response to Hb as demonstrated by SPR and QCM-D experiments (Figure 33).

At very low concentration (0.01 mg ml⁻¹) the anti-Hb biointerface installed on BP-CNF was able to detect significant amounts of Hb, 0.4 ± 0.02 mg m⁻² in SPR (equations 3.1 and 3.2) and 0.5 ± 0.4 mg m⁻² in QCM (Voigt model). The amount of bound Hb increased when the concentration of the target analyte was increased to 0.1 mg ml⁻¹ (1.1 ± 0.05 and 2.0 ± 1.0 m gm⁻² in SPR and QCM, respectively). When 1 mg ml⁻¹ Hb was exposed to the BP-CNF, the adsorbed amount was determined to be 3.4 ± 0.2 (SPR) and 4.6 ± 1.1 m gm⁻² (QCM). The dose-response indicated that the mass of bound Hb to the biointerface on BP-CNF increased as the concentration increased (Figure 33). Differences in adsorbed mass by SPR and QCM were observed since SPR monitors change in refractive index and is not significantly affected by coupled water on the surface whereas the frequency change in QCM-D is sensitive to mass changes from both adsorption and water coupling. Indeed, QCM-D showed a higher adsorbed mass for each concentration compared to SPR, indicating increased bound water at increased Hb concentration. Altogether, both SPR and QCM-D results indicate concentration-dependent response of the biointerface to the target molecule.

The ability of the anti-hIgG biointerface prepared on TOCNF with passivating block copolymer for regeneration on repeated detection of hIgG was studied. Figure 34 presents a schematic illustration of the procedure adopted.
Results and Discussion

Figure 34. Schematic illustration of the preparation of anti-hIgG biointerfaces on TOCNF followed by block copolymer passivation. The biointerface is applied for hIgG detection after which it is regenerated (pH 2, I = 0.5 M). After regeneration, the conjugated anti-hIgG remain on the surface and the biointerface can be applied for hIgG detection again.

First, antibodies were attached on TOCNF via EDC/NHS activation. Then the passivation of non-activated surface was achieved by block copolymer adsorption. After the biointerface was employed for hIgG detection, it was regenerated with acidic washing solution (pH2, I=500mM). After regeneration, the block copolymer was applied again and the biointerface was utilized for hIgG detection once more, followed again by regeneration. The procedure was monitored with SPR in real time (Figure 35) to determine the functionality of the anti-hIgG biointerface.

Figure 35. Three regeneration cycles of the anti-hIgG biointerface on TOCNF with block copolymer passivation. D$_{33}$-b-EGMA$_{137}$ is adsorbed on the biointerface followed by rinsing and hIgG detection. The biointerface is then regenerated (pH2, I=500mM) and rinsed with the respective buffer solution. The cycle is repeated three times. Droplet symbols represent rinsing with 10 mM PB at pH 7.4.
Results and Discussion

Upon regeneration of the TOCNF with acidic wash, an increase in the SPR angle was observed likely resulting from the substantially higher salt concentration of the regeneration solution compared to the 10 mM PB used (0.5 M vs. 0.025 M) (Yunus, Rahman 1988). When the respective phosphate buffer solution was changed back, the baseline returned to approximately same level as prior to block copolymer adsorption (-0.05 °), indicating successful regeneration of both the D_{33}-b-EGMA_{137} copolymer and hIgG. The slightly lower SPR angle value compared to the initial baseline might result from removal of some residual electrostatically adsorbed anti-hIgG from the surface. Some electrostatically bound antibodies could have remained on the surface despite the ethanolamine treatment and were now removed with the very harsh, low pH, salt washing. The fact that only ~65% of initially adsorbed amount of hIgG could be recovered in the following detection and regeneration cycles further supports this assumption.

However, generally the regeneration experiments confirmed that the antibody-TOCNF biointerface with block copolymer passivation could be regenerated and applied with good reproducibility for hIgG detection at least three times. The regeneration of anti-hemoglobin biointerface prepared on BP-CNF was also explored by adsorbing 1 mg ml⁻¹ hemoglobin on the biointerface followed by regeneration with HCl-glycine solution (pH 2.5). Each regeneration cycle step was imaged with AFM (Figure 36).

![AFM images](image)

Figure 36. AFM images (1×1 μm², z = 14 nm) for reference BP-CNF(a) anti-Hb biointerface installed on BP-CNF (b), Anti-Hb biointerface on BP-CNF with detected Hb before (c) and after (d) regeneration. Include is also SPR sensograms for 1 mg ml⁻¹ Hb (10 mM PB, pH 7.4) adsorption onto anti-Hb biointerface prepared on BP-CNF (e).

The AFM image for the reference BP-CNF surface revealed a fibrillar structure with an RMS roughness of 1.7 nm (Figure 36a). The fibrillar structures became less visible upon anti-Hb conjugation and globular features appeared on top of the fibrils, likely originating from aggregated proteins during drying of the samples. The reported dimensions for IgG molecules are 14.5×8.5×4.0 nm (Lee et al. 2002) i.e., they are significantly smaller than the lateral dimension of BP-
CNF fibrils and too small to be observed individually in AFM. The RMS roughness decreased somewhat to 1.6 nm upon anti-Hb conjugation, indicating filling of voids and interfibrillar spaces by the proteins and their aggregates.

The binding of Hb by the anti-Hb biointerface on BP-CNF resulted in the observation of distinct globular structures on top of the biointerface. The dimension for the ellipsoid-shaped hemoglobin is reported to be $6 \times 5 \times 5$ nm (Erickson 2009), which suggests that the observed globular features were Hb proteins that aggregated upon adsorption and subsequent drying of the sample. A distinct increase from RMS roughness of 1.6 nm to 2.3 nm upon Hb binding was observed, further validating the findings. Qualitative information about the regeneration ability of the biointerface can be achieved with AFM. Indeed, upon HCl-glycine treatment the RMS roughness of the biointerface returned to a level similar to that prior to Hb binding (1.8 nm) and the distinct globular structures diminished and the surface resembled more that of BP-CNF with anti-Hb biointerface (Figure 36b). SPR measurements further confirmed the successful regeneration of 1 mg ml$^{-1}$ Hb of the anti-Hb biointerface on BP-CNF upon HCl-glycine treatment (Figure 36e).

4.3.4 Adjusting sensitivity and selectivity of biointerface

One way to improve biosensor sensitivity is to utilize oriented immobilization of capture analytes that maximize the available binding sites of target analyte. In this work, protein A from *Staphylococcus aureus* was adopted to create an oriented antibody biointerface on TOCNF. Protein A is capable of binding the Fc portion of IgG molecules of various species without disrupting the antigen recognition capabilities of the antibody (Boyle, Reis 1987). The hypothesis here was that protein A would increase the favorable orientation of anti-hIgG (Fab fragments upwards toward the solution) on the TOCNF surface and thus increase hIgG detection sensitivity. The difference in adsorbed amount of hIgG by non-oriented and oriented anti-hIgG biointerface on TOCNF is presented in Figure 37, together with a schematic representation of the non-oriented and oriented biointerface with protein A.

![Figure 37](image)

**Figure 37.** Adsorbed amount of hIgG on non-oriented and oriented anti-hIgG biointerfaces prepared on TOCNF and schematic illustration of the difference between the randomly oriented and oriented biointerface with protein A.
The oriented anti-hIgG biointerface was prepared similarly as the non-oriented one, but instead of anti-hIgG, protein A was conjugated onto TOCNF with EDC/NHS. Anti-hIgG was then adsorbed onto protein A and the surface was passivated with D₃₃-EGMA₁₃₇. The binding of hIgG on the oriented biointerface was then tested, which resulted in 43% increase in hIgG detection compared with the non-oriented anti-hIgG biointerface (adsorbed amounts of 1.28 ± 0.11 and 0.90 ± 0.09 mg m⁻², respectively). The increased sensitivity of the oriented biointerface is likely the result of better orientation of the anti-hIgG molecules (Figure 37). Protein A has affinity for the non-antigen-binding Fc region of antibodies leaving the antigen-binding Fab fragments facing towards solution for more efficient hIgG binding. In contrast, for the non-oriented biointerface antigen-binding sites of randomly attached anti-hIgG might be unavailable due to steric hindrance. The reported hIgG adsorbed values were good compared to other anti-hIgG biointerfaces prepared on cellulosic materials (Orelma et al. 2012a, Orelma et al. 2012b) and to other hIgG binding surfaces prepared on alkanethiol self-assembled monolayers (Islam et al. 2014a, Islam et al. 2014b) and grafted copolymer brush surfaces (Zhang et al. 2012a) and comparable for those reported for other antibodies (Carrigan et al. 2005a, Chung et al. 2012).

The anti-Hb biointerface was prepared on BP-CNF to investigate the interference of the BP activation with conjugation of antibodies onto the material and the detection of target molecules. As discussed earlier, the effect of BP functionalization on conjugation efficiency of antibodies was found to be low, indicating little disturbances from the benzophenone functionalization. In addition, the prepared antibody biointerface was found to be highly specific for hemoglobin, as tested by non-specific adsorption of HSA. However, as also discussed previously, the non-specific adsorption of the target analyte itself (in this case Hb) can cause problems in accurate biosensor development and results in false positive responses. However, during biointerface preparation the surface was to some extent blocked already by the conjugated capture antibody, reducing non-specific adsorption significantly due to surface saturation. With this in mind, we investigated also the possibility of tuning the properties of the developed material platform of BP-CNF in desired directions i.e. sensitivity and selectivity of the biointerface. Random copolymers consisting DMAEMA and OEGMA (D₄₆-rnd-EGMA₃₉₉) were found to be excellent in reducing non-specific adsorption of HB onto BP-CNF (Paper IV) and were therefore employed for anti-Hb biointerface preparation (Figure 38). The total blocking of non-specific Hb adsorption on BP-CNF biointerface decreased the sensitivity of the detection, as expected.
Results and Discussion

Figure 38. SPR sensogram for Hb detection (0.1 mg ml⁻¹) by anti-Hb biointerface prepared on BP-CNF and after the application of random copolymer blocking agent (a). Comparison of detected amount of Hb at different concentration (0.01, 0.1 and 1 mg ml⁻¹) by the anti-Hb biointerface on BP-CNF with (red) and without (black) blocking determined with equations 3.1 and 3.2.

SPR was used to monitor Hb adsorption on the anti-Hb biointerface prepared on BP-CNF, after blocking with of D₄₀-rnd-EGMA₁₃₉ (Figure 38a). The experiments indicated that high levels of Hb (0.59 ± 0.07 mg m⁻², c(Hb)=0.1 mg ml⁻¹) could still be detected with extremely effective blocking agent applied. The anti-Hb biointerface on BP-CNF with surface passivation also resisted non-specific adsorption of HSA similarly as the one without blocking agent applied. This confirmed that the biointerface retained high specificity for Hb while the selectiveness increased (Paper IV). Overall, the adopted methodology allowed for highly selective biointerface preparation on BP-CNF towards Hb with high specificity, efficient surface passivation and reduced false response.

The response of the anti-Hb biointerface with effective blocking for Hb concentration concentrations (0.01, 0.1, and 1 mg ml⁻¹) was tested similarly as for the non-blocked biointerface. With the lowest concentration 0.01 mg ml⁻¹, similar levels of Hb binding were reported for both with and without blocking (0.43 ± 0.02 and 0.41 ± 0.02 m gm⁻², respectively). At higher Hb concentrations the Hb detected amount was somewhat decreased with the blocking agent applied on the biointerface, as expected (Figure 38b). The results indicate that the properties of the prepared biointerface on BP-CNF can be tuned to enhance the biointerface. The biointerface can be tailored to be highly selective and specific for Hb with a small penalty in sensitivity, which is the trade-off that exists between the two properties.

4.4 Biosensor development on nanocellulose filaments

The studied methodologies to modify properties of TOCNF for utilization in biomedical or diagnostic applications were employed to demonstrate biosensor development on TOCNF materials. The obtained results with model film studies were translated to cellulose filaments. Wet-spun BP-CNF were utilized for ELISA type of testing of Hb from solution with fluorescent signaling. Figure 39
presents a schematic illustration of the wet-spinning of filaments as well as the biosensor preparation steps on BP-CNF filaments.

**Figure 39.** Schematic representation of the wet-spinning process and UV activation for water-resistant BP-CNF filaments followed by EDC/NHS activation and conjugation of anti-Hb i.e. anti-Hb biointerface preparation with subsequent Hb detection and reporting of the detection event with fluorescein-labelled secondary anti-Hb antibody.

The 1D structure of the BP-CNF filaments offered unique opportunities for the utilization of this bifunctional material with water-resistance and bioactivity. Non-traditional and emerging areas of biomedical applications, such as implantable diagnostic devices (Mostafalu et al. 2016), cell therapy (Mertaniemi et al. 2016), and tissue engineering (Tamayol et al. 2013) can benefit from associated materials. The water-resistance of the material allowed for easy handling in aqueous environments while the bifunctional properties facilitated the modification to capture specific targets. Compared to traditional test strips and lateral flow assays the developed material platform offered easier multiplexation without need for hydrophobic channels. The material was naturally flexible but robust and, importantly, sufficiently water-resistant to eliminate the need for plastic support, improving overall material efficiency. Furthermore, the filaments were regenerable as demonstrated by the SPR and QCM-D results presented previously, they are made from renewable material, and fully biodegradable to offer many advantages compared to traditional supports. As a demonstration of the potential of this developed bifunctional material for these purposes, the BP-CNF filaments were deployed for ELISA type of rapid recognition of Hb levels in aqueous media, which can be indicative of many physiological conditions, such as hematuria or hemoglobinuria (Simerville et al. 2005).

After the wet-spun BP-CNF filaments were extruded and dried, anti-Hb was conjugated onto them with EDC/NHS similarly as for the anti-hIgG biointerfaces on BP-CNF model films (Paper IV). Subsequently, the filaments were employed for Hb detection from solution at different concentrations (0.01, 0.1 and
1 mg ml⁻¹) followed by reporting of the successful detection with an FITC-labelled secondary anti-Hb antibody. The resultant filaments were imaged with confocal scanning laser microscopy (Figure 40).

![Figure 40. Confocal laser scanning microscope image of BP-CNF filament (a), reference anti-Hb biointerface installed on BP-CNF without Hb detection (b) and anti-Hb biointerface installed BP-CNF filaments with detected Hb (1 mg ml⁻¹) and reporting with FITC-labelled secondary anti-Hb antibody.](image)

The ELISA-principle with fluorescence reporting was adopted to demonstrate the rapid recognition of Hb from solution by the BP-CNF filaments as indicated previously for the BP-CNF model films. CLSM was used to monitor changes in the fluorescence in each step of the biosensor preparation. Detailed descriptions of the experiments and discussion is provided in Paper IV. The Hb-binding on BP-CNF filaments was prepared similarly as for the model film studies where EDC/NHS chemistry was utilized to conjugate anti-Hb (0.5 mg ml⁻¹) on the surface. The binding of Hb (1 mg ml⁻¹) on the BP-CNF biointerface was then tested followed by reporting of the possible capture event by secondary anti-hIgG antibodies that were labelled with fluorescein.

Some auto-fluorescence was observed on the reference BP-CNF filaments (Figure 40a) due to the grafted benzophenone groups (Brown et al. 1972). After conjugation of anti-hIgG on the BP-CNF surface, no detectable changes were observed in auto-fluorescence of the samples (Figure 40b). After the detection of Hb from solution and attachment of the secondary reporting antibody with FITC, a distinct increase in fluorescence of the BP-CNF filament was observed (Figure 40c). Hb concentration-dependent fluorescence response was observed for the biointerface prepared on BP-CNF (Paper IV), indicating the potential of the system for quantitative analysis. Although clearly observable, the relatively small increase in fluorescence upon secondary FITC-labelled antibody adsorption might be due to the fact that according to conductometric titration 70% of the carboxyl groups of TOCNF was already utilized in preparation of BP-CNF. Therefore, the available sites for EDC/NHS activation and antibody conjugation on BP-CNF are scarcer than on TOCNF resulting perhaps in somewhat lower observed fluorescence signal. Overall, the experiments here and previously demonstrated a bifunctional material platform with possibility for regeneration on BP-CNF that can be utilized for rapid biomolecule detection or affinity adsorption. The material provides excellent opportunities and potential for emerging and novel diagnostic applications, such as implants or multiplex assays with
improved material efficiency. They are potentially environmentally friendly, sustainable alternative material compared to plastic based dipsticks, and the general nature of the conjugation chemistry presented here open possibilities for a wide range of introduced functionalities and utilization targets.
5. Concluding Remarks

The objective of this work was to develop new multi-functional material platforms by introducing bioactivity on cellulose nanofibrils. First, the effect of block and random copolymer adsorption on material properties was investigated for cellulose substrates carrying different charges. Copolymers containing highly hydrophilic PEG segments endowed cellulose with antifouling effects. Adsorption of PDMAEMA-POEGMA block and random copolymers on cellulose surfaces of various charge densities was investigated. The adsorption was found to be mainly driven by electrostatic interactions between anionic cellulose and positively charged segments of the copolymers. Moreover, peculiar QCM frequency shifts observed upon block copolymer adsorption onto TOCNF were determined to result from significant water expulsion from the interface, due to charge neutralization, as was verified by SPR measurements. Compared to the block copolymers, random copolymers presented different adsorption kinetics, with fast initial adsorption followed by reduced but linearly increasing adsorption rate.

PDMAEMA-POEGMA block and random copolymers were found to be excellent in reducing non-specific human IgG adsorption onto TOCNF. As a result, 84-100% reduction in non-specific binding was determined by SPR, further supported by AFM imaging. Moreover, while the block copolymers were highly efficient in producing antifouling TOCNF surfaces, they did not limit the affinity and bioactivity to target molecules via anti-hIgG, which was installed on TOCNF through EDC/NHS coupling. Such biointerfaces exhibited good levels of hIgG detection from solution while block copolymer passivation prevented a nine-fold false response caused by non-specific adsorption. This highlights the importance of adequate surface passivation in biosensor development from cellulose-based materials. Antifouling is extremely important in developing materials with potential for tissue embedding and implantation.

The attachment of functional molecules onto CNF from aqueous media is feasible only through activation of cellulose, given the relative inertness of the hydroxyl groups in polar media. Such strategies, including TEMPO-oxidation, may come at the expense of lowered wet mechanical strength that is otherwise crucial in any deployment, especially in the biomedical area. In order to gain the necessary surface activation through TEMPO-oxidation, while introducing better wet strength, TOCNF was conjugated with photoreactive benzophenone through EDC/NHS coupling, to obtain BP-CN. Upon UV activation, the conjugated benzophenone groups crosslinked with cellulose and a gain in wet
strength of the material was realized, as determined via tensile testing. Successful attachment of benzophenone was determined with XPS analyses and conductometric titration. Moreover, water-resistant filaments of BP-CNF were prepared through wet-spinning. A remarkable retention of 84% of the initial dry strength was achieved. Additionally, the filaments exhibited bifunctional properties since the potential for further activation through a second EDC/NHS coupling was not significantly impaired by the presence of benzophenone. Indeed, an anti-Hb biointerface prepared on BP-CNF exhibited an excellent ability for Hb detection, with negligible non-specific adsorption (HSA), as demonstrated in SPR and QCM-D. Also, the sensitivity and selectivity of the anti-Hb biointerface was tuned by applying PDMAEMA-POEGMA random copolymer blocker. Finally, the wet-spun BP-CNF filaments with an anti-Hb biointerface were successfully employed for Hb detection from solution by reporting with fluorescence-labelled secondary antibody, as determined by CLSM.

Altogether, this thesis presented new understanding related to surface interactions of nanocellulosic materials and offer novel methods to modify their surface properties for utilization in emerging biomedical applications. The developed methodology endowed native cellulose filaments with superior mechanical strength in the wet state. Remarkably, this did not interfere with the potential of the material for bioactivation, which opens opportunities in tissue engineering, biosensor development, implantable devices, tissue embedding and drug delivery. These observations go beyond traditional 2D films since desired material properties were successfully accomplished on filaments that further widen the potential range of uses. Enabling TOCNF with extreme protein resistance, expand the possibilities for novel applications such as implants and accurate biosensors, given the fact that copolymer passivation does not interfere with antigen-antibody interactions. Overall, the results of this work offer a better understanding of the interactions and properties of cellulose nanofibrils with biomolecules and could potentially open avenues for a wide range of sensor platforms. Based on the work presented in this thesis, future studies in could focus on investigating the longevity of the anti-fouling coatings, developing better reporting systems, and exploring the potential of tissue embedding.


References


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