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Dissertation
102

# Biochemical modification and functionalization of nanocellulose surface

Suvi Arola

VTT

VTT SCIENCE 102

# Biochemical modification and functionalization of nanocellulose surface

Suvi Arola

*A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Science, at a public examination held in the lecture hall TU1 (Otaniementie 17, Espoo) of the school on 15th of August 2015 at 12 noon.*

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# Abstract

Cellulose is an abundant biopolymer found in many different organisms ranging from microbes to plants and animals. The homopolymer, composed of repeating glucose units, forms mechanically strong nanosized fibrils and rods. In plants cellulose forms macroscopic fibers, which are incorporated in the cell walls. Recently, it has been shown that cellulose fibers can be disintegrated into the fibrils and rods by different chemical treatments. These materials are called nanocellulose. Nanocellulose is a promising material to replace fossil based materials because it is renewable, biodegradable and abundant. It holds great potential in many applications due to its superior mechanical properties and large surface area. For most applications modification of nanocellulose surface is needed due to its tendency to aggregate by hydrogen bonding to adjacent cellulose surfaces.

In this thesis we took a biochemical approach on nanocellulose surface modification to achieve modified and functional materials. The advantages of this approach are that the reactions are done in mild aqueous ambient conditions and the amount of functionalities of biomolecules is broad. Four different approaches were chosen. First, genetically engineered cellulose binding proteins, were used to introduce amphiphilic nature to nanocellulose in order to create surface self-assembled nanocellulose films and to stabilize emulsions. This method was shown to be a good method for bringing new function to nanocellulose. (Publication I) Second, covalent coupling of enzymes directly onto modified nanocellulose surfaces provided a route for protein immobilization in bulk. Nanocellulose derivatives were shown to be well suited platforms for easy preparation of bioactive films. More over the film properties could be tuned depending on the properties of the derivative. (Publication II) Third, by modifying the nanocellulose surface with specific enzymes we could study the role of hemicellulose in nanocellulose fibril surface interactions. We showed that hemicellulose has an important role in nanofibrillated cellulose networks, yet its effects were different in aqueous and dry matrixes. (Publication III) Fourth, by modifying the specific function of cellulose binding protein via genetic engineering we showed how the binding properties can be altered and thus the functionalization properties can be tuned, and that the cellulose binding protein properties are substrate dependent. We also showed that nanocellulose as a model substrate in binding studies is a valuable tool for gaining new insight in protein binding behavior. (Publication IV)

In conclusion, we showed that biochemical methods are feasible in nanocellulose modification and functionalization to study intrinsic properties of nanocellulose and cellulose binding proteins but also for creating new functional materials.

# Tiivistelmä

Selluloosa on laajalti luonnossa esiintyvä biopolymeeri, jota tuottavat kasvien ja mikrobien lisäksi jotkin eläinkunnan jäsenet. Tämä homopolymeeri, joka muodostuu toistuvista glukoosiyksiköistä, muodostaa mekaanisesti vahvoja nanokokoluokan fibrillejä ja tikkuja. Kasveissa selluloosa muodostaa mikro- ja makrokokoluokan kuituja, jotka ovat osana soluseinärakennetta. Hiljattain on pystytty näyttämään, että selluloosakuidut voidaan hajottaa fibrilleiksi ja tikuiksi erilaisin kemian menetelmin. Näitä materiaaleja kutsutaan nanoselluloosaksi. Nanoselluloosa on lupaava materiaali korvaamaan öljypohjaisia materiaaleja, koska se on uusiutuva, biohajoava ja helposti saatavilla oleva. Nanoselluloosa on myös potentiaalinen materiaali monilla eri teknologioiden aloilla suuren pinta-alansa ja ylivertaisten mekaanisten ominaisuuksiensa takia. Suurin osa sovelluksista vaatii nanoselluloosapinnan ominaisuuksien muokkaamista, koska nanoselluloosakuidut liittyvät helposti yhteen vetysidosten välityksellä.

Tässä väitöskirjassa on tutkittu biokemiallisten menetelmien soveltuvuutta nanoselluloosapintojen muokkaamisessa ja funktionalisoimisessa. Näiden menetelmien etuja ovat, että reaktiot tapahtuvat miedossa vedellisessä ympäristössä ja että biomolekyylien toiminnollisuuksien joukko on erittäin laaja. Valitsimme neljä erilaista lähestymistapaa. Ensinnä käytimme geneettisesti muokattuja selluloosaan sitoutuvia proteiineja tuomaan amfifiilisyyttä nanoselluloosan pinnalle. Näitä rakenteita käytettiin muodostamaan itsestään järjestäytyneitä pintoja nanoselluloosasta sekä stabiloimaan emulsioita. Tämän menetelmän näytettiin toimivan nanoselluloosan funktionalisoinnissa. (Osajulkaisu I) Toiseksi näytimme, että nanoselluloosan johdannaiset toimivat hyvin matriisina proteiinien suoralle kovalenttiselle kiinnittämiselle bulkissa. Nämä johdannaiset sopivat hyvin bioaktiivisten filmien valmistukseen. Lisäksi filmien ominaisuuksia voitiin muuttaa ja muokata nanoselluloosajohdannaisen ominaisuuksista riippuen. (Osajulkaisu II) Kolmanneksi tutkimme hemiselluloosan roolia nanoselluloosamatriisissa muokkaamalla nanoselluloosan pintaa spesifisillä entsyymeillä. Pystyimme näyttämään, että hemiselluloosalla on tärkeä rooli näissä verkostoissa, joskin se on erilainen kuivissa ja kosteissa systeemeissä. (Osajulkaisu III) Neljänneksi, muokkaamalla geneettisesti selluloosaan sitoutuvan proteiinin spesifiä toimintoa, näytimme, että pystymme vaikuttamaan sen sitoutumisominaisuuksiin ja näin mahdollisesti myös nanoselluloosamateriaalien toiminnollisuuteen. Näytimme myös, että selluloosaan sitoutuvan proteiinin toiminta on riippuvaista sen substraatista ja että nanoselluloosa on hyvä mallisubstraatti sitoutumiskokeissa, sillä se tuo uutta tietoa näiden proteiinien toiminnasta. (Osajulkaisu IV)

Yhteenvetona voi todeta, että erilaiset biokemialliset menetelmät soveltuvat nanoselluloosapinnan muokkaukseen ja funktionalisointiin, nanoselluloosan ja siihen sitoutuvien proteiinien luontaisten ominaisuuksien tutkimisessa, mutta myös uusien toiminnallisten materiaalien luomiseen.

# Preface

The practical work of this Ph.D. thesis was carried out at VTT Technical Research Centre of Finland Ltd during the years 2010–2015. I warmly thank current and former technology managers Dr. Kirsi-Marja Oksman-Caldentey, Dr. Raija Lantto, and Dr. Niklas von Weymarn, and especially the Vice President R&D Professor Anu Kaukovirta-Norja for providing excellent working facilities and equipment. I express my gratitude to Professor Liisa Viikari, the director of the former BIOREGS – Biomass Refining Graduate School, who accepted me as a Ph.D. student and provided a top-notch scientific environment where to conduct my thesis. BIOREGS, Tekes – The Finnish Funding Agency for Innovation, Academy of Finland, Academy of Finland's Centre of Excellence HYBER, and Emil Aaltonen's foundation are acknowledged for funding the work.

I thank my pre-examiners Professor Kiyohiko Igarashi and Dr. Tanja Zimmermann for evaluating my thesis and for giving me valuable comments. I also want to thank my opponent, Professor Neel S. Joshi, for accepting this task and coming to Finland to examine my work.

I want to express my gratitude to Academy Professor Olli Ikkala who took me into his Molecular Materials group at the Department of Applied Physics in 2010 to do my Ph.D. work. My first thought was: "A molecular biologist in an applied physics group... What's going on?" But I quickly learned, that although the "walls were high, the sealing was open" in the MolMat group. And I enjoyed this privilege of freedom very much. I'm also very grateful for Professor Ikkala for his support throughout my studies and his enthusiasm and passion for science that feeds the great research done in his group.

I want to acknowledge Dr. Kvar Black for reading the thesis and correcting my English on such a short notice. Thank you Mr. B! I don't think anyone else would have done the job better than you!

I have had the privilege of working together with the BEST of the best supervisors, Professor Markus Linder. I cannot imagine a better teacher in science, academy, or life for that matter than him. During these years Markus has become more of a mentor and friend than a boss and supervisor for me. I have too many things to thank him for but to list a few I'll start by thanking Markus for initially taking me into

his Nanobiomaterials group at VTT to be a researcher. That was where it all started from! I'm forever thankful for Markus for giving me a chance to chase my dream, to become a Ph.D., and finding the absolute best way to do it; as an Ext at VTT. Markus, you have always had the time for me and the patience to listen and guide me to the right direction, yet, giving me the freedom to learn and be independent. Your enthusiasm and motivation for the science has been the best fuel for this Ph.D. work. I've always felt that I've had your 150% support throughout the whole time. (You even had tissues in your office for the times I wasn't doing so well in the lab, not to mention the very last weeks of the writhing process of this thesis! Okay, maybe the tissues weren't only for me, but still…) I'm grateful for the open and warm working environment that you've provided. It has had a great impact on the work and also made me feel that no matter what, you'll stand behind my back and take a catch if needed. Thanks to Markus, I'm also confident that I'm a better storyteller now than I was five years ago. I also know now that such terms like "quick and dirty" do not apply in science, at least the "quick" part of it. Yet, what is quick, remains to be defined and might vary depending on one's academic status. J All this is only a tip of the iceberg and there are so many other things I would need to say here, but to keep it short… Markus, thank you for everything, really, everything! This thesis would not be as good as it is without your commitment and dedication!

Over the years I have had the pleasure of working with absolutely the best and most skilled experts. I want to thank all my co-authors (Prof. Päivi Laaksonen, Dr. Timo Laaksonen, Dr. Hanna Valo, Dr. Hendrik Hähl, Dr. Arja Paananen, Dr. Tekla Tammelin, Dr. Harri Setälä, Antti Tullila, Dr. Jani-Markus Malho, and Dr. Martina Lille) and colleagues for helping me in getting this work done. Without all of you this thesis would not exist! Especially, I want to thank Dr. Tekla Tammelin for helping me understand the surface chemistry of nanocellulose and for her expertise in this field. I enjoyed our collaboration and discussions very much! I thank Dr. Martina Lille for her excellence and knowledge in the field of rheology and specifically rheology of nanocellulose. I would have been lost many times during this work without you! I want to express my warmest thanks to all my former group members (Nanobiomaterials and Protein Discovery and Engineering) at VTT. You made me feel accepted and welcome, and coming to work every day was a great joy simply because you were there. I want to thank Dr. Riitta Partanen for being such a great team leader for the Nanobiomaterials group promoting the excellent team spirit we had. You were always open for discussion and I felt that you really cared for us. You also did a great job in bringing people from different disciplines together and making the team really well functioning.

In addition I express my deepest gratitude to Dr. Arja Paananen who initially introduced me to VTT and to most of the techniques I used in this thesis. I must say, it was quite tough on me learning so much during so little time but I also enjoyed it very much, thanks to you! You have not only been a great colleague and a great scientific support to me but also a great friend too! I really miss working together with you! I want to thank Professor Päivi Laaksonen for the great scientific input on my work. We've had a lot of fun working together and I'm grateful for all the help and guidance I received from you during this work. I really look up to you as a great

role model. I'm also very happy that we have become great friends and that our boys enjoy the time spent together as much as we do. J I want to express my gratitude to Dr. Dilek Ercili-Cura for introducing me to the secrets (and pain) of surface shear rheology and teaching me how to use the AR-G2. The sometimes frustrating days at the rheometer were more fun with you than alone! I thank Dr. Géza Szilvay especially for helping me a lot in the genetic engineering part of this work. You have always been so very helpful in every possible way. I enjoyed our scientific discussions and also the not-so-scientific ones! I thank Arja Kiema for excellent assistance with the *Trichoderma* work and protein production. You and Géza took the work forward during my maternity leave, and I'm very grateful for that. I want to express my warmest and deepest gratitude to Riitta Suihkonen, our "mom" in the small lab, for being such a big help during the experimental work. But you have been more than just technical help, you are a great and dear friend to me! You were always there for me (in the ÄKTA room) to listen, laugh, gossip, cry, hug, and celebrate before, during and after work! I really miss our everyday chit-chat so much! I really miss you!

I left VTT at the end of the year 2014 and went on to Aalto Biomolecular Materials group (lead by Markus). And I have to say… What a group!! We have such great people doing such great science! All of you: Markus, Heli, Georg, Katja, Bartek, Bart, Chris, Wenwen, and Pezhman I want to thank you for making the group what it is! You make my day every day when I come to work. Especially I want to thank our office crew at D332a; Heli, Georg, Katja and Bartek. I've laughed so much with you during the past months and we have had so much fun that I couldn't imagine a better office! You kept me going and encouraged me during the writing process, you shared your joys and sorrows with me, and you always went for coffee with me even though you probably didn't have the time (I know I certainly always didn't, but it was definitely worth it). I will miss you guys dearly when I move out!

My friends, You truly are the best and I'm privileged to have you in my life! Johanna, I want to thank you for sharing the great joys and bumps on the way of becoming a Ph.D.! You have picked me up with your encouragements when I felt that nothing was working and I'll just quit! You have shared the celebrations of "today the stuff worked in the lab" because you really know that it might not be that great tomorrow! We've always had the best scientific discussions on what's really going on with nanocellulose since it isn't that trivial. But you have not only been a great peer support in science but also as a mom! That is priceless! Kaisa, we've known since undergrad times from Chem, and no one else was as crazy and as enthusiastic about chemistry as you! I think I have to blame you I survived as long as I did there. We've had so much fun over the years! Come on… who else would order a GT in a bar (with UV-lights on the dance floor) and make an experiment with salt!! I think we were labelled as geeks at that bar! J Alise, my dear friend from Tampere. We studied together at IMT and shared more than just the passion for bio… the passion for shoes, purses and Miyazaki films! I'm so glad I met you then, you really made the endless hours of studying more fun! You also introduced me to Tampere and made the city feel like home for me! You really mean the world to me! Kim, although life has taken us sometimes far away from each other physically, I've

always felt that you've been very close to me. That, I guess, happens when you've known someone most of your life. We've known for 30 years! You enrich my life with beauty and spirituality that you radiate! You are so special to me! Maria, my dear, dear, Maria! I simply love you! There is no one else who makes me laugh like you, or cry like you! To you, I can reveal all my secrets and I know you won't judge me. You're positivity, kindness and great selflessness never seems to stop amaze me! The girls' gourmet club (Maria, Iida, Arnevi, Anna J., Anna K., Anmi, Katja), what would I do without you!? Food (good wines) is a great way (good excuse) to escape the ordinary life and have fun! I have no words to describe the gratitude I feel for having such great people in my life! Growing up with you has made an impact on me that can only be described with the most positive and fun adjectives! I love you all so much! Leandro, you were the one who encouraged me to pursue my dream of getting a Ph.D., and you did it with your own example! I'm so lucky that I got to know you! Thank you for sharing science and life with me! Sanja, where do I start!? Thank you for sharing the ups and downs of owning and loving dogs. Thank you for sharing the passion for animal welfare and nature! Thank you for sharing the passion for being an activist! J Thank you for the countless hours in the middle of Espoo forests and endless discussions on how we would make the world a better place! Thank you for being a part of my life! Thank you for being exactly what you are! Anna-Maria and Pekka, I want to thank you both for your hospitality that you have shown for my family and me for the past years! You are also to blame for our Hugo. Without you, I'm certain that we would not have thought of getting a coated hairless dog. J I want to thank Pekka for my first "real" job after I finished my undergraduate studies. Finnzymes was certainly a great place to work, like a big family! You, with your wise and firm but warm mentality, made the environment absolutely remarkable! Anna-Maria, thank you for introducing me to Peruvian hairless and their mysteries! But also you have introduced me to my second home, the back forests of Espoo and the great dog hobbies we have! That is priceless! Thank you for being such a great friend, and a great support in all aspects of life!

My family, the whole lot of you! I'm so lucky to have you all. I have two great sister-in-laws who full my life with laughter and joy. Thank you Milma and Varja for being a part of my life. In addition I want to thank Jaakko (Varja's husband) for making sure that Alari and I get to eat also other food than just mashed potatoes and meatballs! J I want to thank my dear sister, Saija, for being in my ilfe. You are the best sister in the whole world. The completion of this thesis would have not been possible without the help and support from my parents and my in-laws. I want to express my warmest regards and deepest gratitude to my mother and father in-law, Hilkka and Kari. You have been more than kind to me always. You have taken me in to your home and family and supported us and me no matter what! Hilkka, you truly are a supper woman, being able to fix everything and anything, having the longest nerves I ever seen and being able to stay calm no matter what happens. I admire you for that! Kari, I want to thank you for countless kilometers that you have been driving for us, bringing forgotten items to work, picking up kids from hobbies, rushing to the supermarket just to get us something we thought we had but didn't… Even more, I want to thank you for being you, our pappa, a very dear and special

person for all of us, especially the kids. I want to thank my father (isi), Olli, and his wife, Jutta, for all the love and support. You have always trusted my judging and supported me on every quest I've gone for in my life! Isi, you have always been my hero, and continue to be. You have always been by my side and you have always defended me if needed. I love you so much! My mom (äiti), Outi, and her husband, Tom, I am forever grateful for your endless support in science and life. No matter what has come in front of me, I've always known where to get help and where to go! Your door has always been open for me. I'm grateful for the offerings of your kitchen when I was a student with little money and a fridge with merely a light in it. I want to thank Tom for being the best personal tutor in physical chemistry during my chemistry studies. I owe you for those top grades! Äiti, I want to thank you for giving me a personal view to what it is to be a researcher and showing me how wonderful it can be. I love you both so much!

My husband, Alari, I cannot begin to describe how much you mean to me. Saying "I love you" is simply not enough. Without your unquestioning support and love this thesis would not exists. You took care of the kids and the dogs while I was working, especially the last year of my Ph.D. must have been tough on you, not to mention the last three weeks or so when you were practically a single parent. And now, you've even agreed to move to the other side of the globe for me! Thank you for being my rock! I thank you for giving me "my everything", my family. Through Alari I have had the privilege of getting to know the sweetest 11-year old girl in the world, Nona! Whom I love very much. Nona has taught me to be an adult, and to let go of my silly fears and fixations. Nona, you are a very special girl! We have two very special personas in the family, Hugo and Irinka, my furry babies. They keep my feet to the ground and my head in the forest!

Finally, I want to dedicate this book to my son, the love of my life, Tuukka! You take me to adventures (usually involving a car drive) every day. The very moment I arrive home you make me forget all my worries and life is perfect. You have shown me what means unconditional love, you have taught me more than any school I ever gone to has, and you have put things into perspective for me. I really don't have to worry about small stuff anymore! I feel proud and privileged to be your mom.

Somewhere over the Atlantic, 10th June 2015
Suvi Arola

## Academic dissertation

| | |
|---|---|
| Supervising professor | Professor Markus Linder<br>Department of Biotechnology and Chemical Technology<br>Aalto University, Finland |
| Thesis advisors | Associate Professor Kiyohiko Igarashi<br>Department of Biomaterials Sciences, Graduate School of Agricultural Sciences<br>The University of Tokyo, Japan |
| | Head of Applied Wood Materials Laboratory Dr. Tanja Zimmermann<br>Applied Wood Materials, EMPA, Switzerland |
| Opponent | Associate Professor Neel S. Joshi<br>Wyss Institute, School of Engineering and Applied Sciences<br>Harvard, Boston, MA, USA |

# List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers.

I Suvi Varjonen, Päivi Laaksonen, Arja Paananen, Hanna Valo, Hendrik Hähl, Timo Laaksonen and Markus B. Linder. 2011. Self-assembly of cellulose nanofibrils by genetically engineered fusion proteins. Soft Matter, 7, 2402–2411.

II Suvi Arola, Tekla Tammelin, Harri Setälä, Antti Tullila, and Markus B. Linder. 2012. Immobilization-stabilization of proteins on nanofibrillated cellulose derivatives and their bioactive film formation. Biomacromolecules. 13, 594–603.

III Suvi Arola, Jani-Markus Malho, Päivi Laaksonen, Martina Lille, and Markus B. Linder. 2013. The role of hemicellulose in nanofibrillated cellulose networks. Soft Matter. 9, 1319–1326.

IV Suvi Arola and Markus B. Linder. Binding properties of single and double cellulose binding modules reveal differences between cellulose substrate. Manuscript submitted to Nature Chemical Biology.

# Author's contributions

**Publication I**

NFC and CNC were gained from the Finnish Centre for Nanocellulosic Technologies. The proteins used in the study were produced and purified by research assistant. The $^{3}$H-labbellig and the purification of the labelled HFBI-DCBM were planned and conducted by the author (previously S.V., now S.A.). The binding studies were planned, conducted and the results were interpreted by author. The Langmuir-trough experiments were planned by the author together with A.P. and conducted by the author. Results were interpreted by the author. The Langmuir-Schaefer films were prepared by the author. The AFM imaging was done by the author and teaching on the use of the equipment was received form A.P. The AFM images were handled by the author and results were interpreted by the author together with P.L. The surface dilatational rheology experiments on air-water interface were planned and conducted by the author. The results were interpreted by the author together with P.L. The interfacial dilatational rheology experiments on oil-water interface were planned, conducted, and the results were interpreted together by the author and P.L. The emulsion studies were planned, conducted and results were interpreted by P.L., author helped in conducting the experiments. The epifluorescence and confocal microscopy experiments were planned, conducted and results were interpreted by P.L., author helped in conducting the experiments. The drug nanoparticle TEM experiments were planned, conducted and otherwise handled by H.V. and T.L. Ellipsometry experiments were planned, conducted and otherwise handled by H.H. QCM-D experiments were planned, conducted and otherwise handled by A.P. The surface shear rheology experiments were planned and conducted by the author, and results were interpreted by the author with the aid of P.L. The manuscript was written mainly by M.B.L. and P.L. The author actively contributed in the writing process of the manuscript. M.B.L. supervised the research.

**Publication II**

The NFC materials used for the modifications and the TEMPO-NFC were received from the Finnish Centre for Nanocellulosic Technologies. Amine modification of NFC and the characterization of the material (XPS) were done by T.T. The epoxy modification of NFC and the characterization of the material (CP/MAS NMR and Kjeldahl method) were done by H.S. The anti-hydrocortisone antibody and the hydrocortisone-AP conjugate were provided by A.T. The author planned and performed the rest of the experiments and interpreted the all results. The author wrote the manuscript with valuable comments from T.T. under the supervision of M.B.L. The research was supervised by M.B.L.

**Publication III**

The NFC used in the work was received from the Finnish Centre for Nanocellulosic Technologies. The pI9 xylanase was received from Dr. Matti Siika-aho. The author planned, conducted and analyzed the results of total hydrolysis of NFC and pI9 hydrolysis of NFC. The author planned the small deformation rheological experiments and interpreted the results together with M.L. The author conducted the small deformation rheological experiments. The author planned the extensional experiments of the NFC films together with J-M.M. The author prepared the films used in the study. J-M.M. conducted the extensional experiments on the films. The author analyzed the data of the extensional experiments together with J-M.M. The author planned the cryo-TEM experiments and prepared the samples. The cryo-TEM imaging was done together with J-M.M. and the results were analyzed by the author. The author planned and conducted the AFM experiments and analyzed the results. The author planned and conducted DWS experiments. The author analyzed the DWS results together with P.L. The author wrote the manuscript under the supervision of M.B.L. The research was supervised by M.B.L.

**Publication IV**

The author planned the work and wrote the manuscript with the aid of M.B.L. The work was fully conducted by the author, except for protein production carried out by Dr. Michael Bayley, and protein purification and cleavage carried out by Riitta Suihkonen. The results were interpreted by the author with the aid of M.B.L. who also supervised the research.

# Contents

# List of symbols

| | |
|---|---|
| $A_f$ | Free amplitude |
| $A_{sp}$ | Set point amplitude |
| AFM | Atomic force microscopy |
| AGU | Anhydroglucopyranose unit |
| Amine-NFC | Amine modified nanofibrillated cellulose |
| AP | Alkaline Phosphatase |
| a-w | Air-Water |
| B | Langmuir one-site binding isotherm |
| B' | Derivative of Langmuir one-site binding isotherm |
| BC | Bacterial cellulose |
| $B_{max}$ | Maximum binding capacity |
| BMCC | Bacterial microcrystalline cellulose |
| °C | Degrees Celsius |
| $CaCl_2$ | Chemical formula of calcium chloride |
| CBD | Cellulose binding domain |
| CBM-Cel6A | Cellulose binding module of cellobiohydralase II |
| CBM-Cel7A | Cellulose binding module of cellobiohydralase I |
| CBHI | Cellobiohydralase I |
| *cbhI* | Cellobiohydrolase I encoding gene |
| CBHII | Cellobiohydralase II |
| CBM | Cellulose binding module |
| CD | Catalytic Domain |

| | |
|---|---|
| Cel6A | Cellobiohydralase II |
| Cel7A | Cellobiohydralase I |
| $cm^2$ | Cubic centimeter |
| CNC | Cellulose nanocrystals |
| *cryo*-TEM | Cryogenic transmission electron microscopy |
| δ | Phase angle (delta) |
| DCBM | Double cellulose binding module |
| DCBM-12 | Double cellulose binding module with a linker of 12 amino acids |
| DCBM-24 | Double cellulose binding module with a linker of 24 amino acids |
| DCBM-48 | Double cellulose binding module with a linker of 48 amino acids |
| deg | Degree |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DP | Degree of polymerization |
| DVS | Dynamic vapor sorption |
| E | Young's modulus, tensile modulus |
| E' | Dilatational storage modulus |
| E'' | Dilatational loss modulus |
| E*/\|E\| | Dilatational complex modulus |
| *E. coli* | *Echerichia coli* |
| ELISA | Enzyme-linked immunosorbent assay |
| Epoxy-NFC | Epoxy modified nanofibrillated cellulose |
| ESI | Emulsion stability index |
| g | Gram |
| γ | Strain (gamma) |
| G' | Shear storage modulus |
| G'' | Shear loss modulus |
| G* | Shear complex modulus |
| ΔG | Gibb's free energy difference |
| GGC | Golden gate cloning |

| | |
|---|---|
| GPa | Giga Pascal ($10^9$ Pascals) |
| *gpdA* | Glyceraldehyde-3- phosphate dehydrogenase gene |
| $^3H$ | Tritium |
| HCl | Chemical formula of hydrochloric acid |
| HCl-CNC | Hydrochloric acid hydrolyzed cellulose nanocrystals |
| HFBI | Hydrophobin HFBI |
| HFBI-DCBM | Hydrophobin HFBI double cellulose binding module fusion protein |
| HFBI-DCBM-12 | Hydrophobin HFBI double cellulose binding module fusion protein with a linker of 12 amino acids between the binding modules |
| HFBI-DCBM-24 | Hydrophobin HFBI double cellulose binding module fusion protein with a linker of 24 amino acids between the binding modules |
| HFBI-DCBM-48 | Hydrophobin HFBI double cellulose binding module fusion protein with a linker of 48 amino acids between the binding modules |
| $H_2O$ | Chemical formula of water |
| *hph* | Hygromycin resistance gene |
| $H_2SO_4$ | Chemical formula of sulfuric acid |
| Hz | Hertz |
| $K_a$ | Association constant |
| $K_d$ | Dissociation constant |
| $k_d$ | Affinity |
| kDa | Kilo Dalton ($10^3$ Daltons) |
| $KH_2PO_4$ | Chemical formula of monopotassium phosphate |
| kPa | Kilo Pascal (= $10^3$ Pascals) |
| $K_r$ | Partitioning coefficient |
| L | Liter |
| LC | Liquid crystal |
| LB | Langmuir-Blodgett |
| LbL | Layer-by-layer |
| LS | Langmuir-Schaefer |
| m | Meter |

| | |
|---|---|
| MALDI-TOF MS | Matrix-assisted laser desorption/ionization – time of flight mass spectrometry |
| MC | Methyl cellulose |
| MCC | Microcrystalline cellulose |
| µeq | microequivalent |
| MFC | Microfibrillated cellulose |
| µg | Microgram ($10^{-6}$ grams) |
| $MgSO_4$ | Chemical formula of magnesium phosphate |
| ml | milliliters ($10^{-3}$ liters) |
| µm | Micrometer ($10^{-6}$ meters) |
| mm | Millimeter ($10^{-3}$ meters) |
| mM | millimolar ($10^{-3}$ moles per liter) |
| mmol | Millimole ($10^{-3}$ moles) |
| mN | Milli Newton ($10^{-3}$ Newtons) |
| m/z | mass to charge ratio |
| NC | Nanocellulose |
| NFC | Nanofibrillated cellulose |
| $(NH_4)_2SO_4$ | Chemical formula of ammonium sulfate |
| nm | Nanometer ($10^{-9}$ meters) |
| NR | Natura rubber |
| ω | Frequency (omega) |
| OLED | Organic light emitting diode |
| o-w | Oil-Water |
| Pa | Pascal |
| *p*-NPP | Para-nitrophenyl phosphate |
| PCR | Polymer chain reaction |
| PD | Potato dextrose |
| PDS | Particle delivery system |
| PEI | Polyethylene imine |
| PIPPS | Piperazine-N,N'-bis(3-phosphanesulfonic acid) |

| | |
|---|---|
| pmol | Picomole ($10^{-15}$ moles) |
| R | The universal gas constant |
| RH | Relative humidity |
| RP-HPLC | Reversed phase high performance liquid chromatography |
| RP-UPLC | Reversed phase ultra-high performance liquid chromatography |
| σ | Stress (sigma) |
| SDS | Sodium dodecyl sulfate |
| SFB | Succinimidyl-4-formylbenzoate |
| SIEBIMM | Strain-induced elastic buckling instability for mechanical measurements |
| $SiO_2$ | Chemical formula of silicon dioxide |
| Sulfite-CNC | Sulfuric acid hydrolyzed cellulose nanocrystals |
| T | Temperature |
| TEMPO | 2,2,6,6-tetramethylpiperidine-1-oxyl |
| TEMPO-NFC | 2,2,6,6-tetramethylpiperidine-1-oxyl oxidized nanofibrillated cellulose |
| TEV | Tobacco eche virus |
| TFA | Trifluoro acetic acid |
| *T. reesei* | *Trichoderma reesei* |
| w/w | Weight per weight ratio |
| XPS | X-ray photoelectron spectroscopy |
| ∞ | Infinite |
| Ø | Diameter |

# 1. Introduction

Cellulose is an interesting material due to its structural features, biodegradability, and renewability. It is also the most abundant polymer on Earth and thus easily available. For example, it can be found in all plants, some alga, and also bacteria. Figure 1 presents the molecular structure of cellulose polymer.[1] Cellulose is a homopolymer formed by D-anhydroglucopyranose units (AGU). The AGUs are linked together by a β-(1→4) glycosidic bond connecting the C-1 and C-4 atoms of adjacent molecules with a 180° rotation in respect to each other that form the repeating unit of cellulose chains, cellobiose. The length of a native cellulose chain (degree of polymerization, DP) varies depending on the origin and treatment. Each AGU has three hydroxyl groups on C-2, C-3, and C-6 position making cellulose a hydrophilic polymer. Terminal groups on cellulose chains are chemically different. On one end the C-1 OH is an aldehyde and has reducing power and it is therefore called the reducing end. On the other end the C-4 OH is an alcohol and thus it is called the non-reducing end.[2,3]

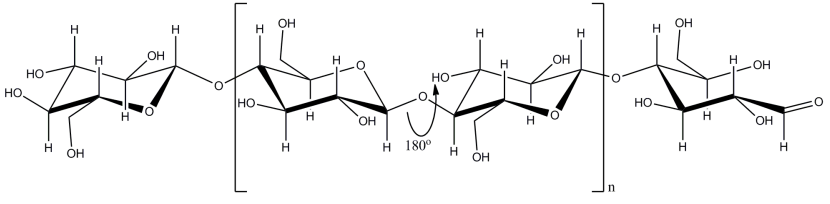


**Figure 1.** Molecular structure of cellulose chain showing the repeating unit, cellobiose, and the reducing (right) and non-reducing (left) end-groups. The AGUs are rotated by 180° with respect to each other.

Cellulose can exist in six different polymorphs; I, II, $III_I$, $III_{II}$, $IV_I$, and $IV_{II}$.[2,4] In nature cellulose is found in the cellulose I polymorph structure where the chains are packed parallel-up and edge-to-edge via extensive intra- and intermolecular hydrogen bonding to form sheet-like structures which form fibril structures.[4] Cellulose I exists in two crystalline forms, cellulose Iα and cellulose Iβ, which have different hydrogen bonding networks.[5–7] The two forms coexist in cellulosic materials but the ratio var-

ies depending on the source. Cellulose Iβ is predominant in higher plants and cellulose Iα in bacteria and tunicate.[7] In the fibrils the crystalline parts are accompanied with less structured amorphous-like regions.[8] The ratio of crystalline-to-amorphous cellulose also varies depending on the source of cellulose. In wood, cellulose is found in the cell wall structures where it is hierarchically packed to form large fibers. These fibers are formed by the stacking of cellulose chains to nanosized elemental fibrils with crystalline and less ordered amorphous parts. These in turn pack into larger nanofibrils that further pack into micron sized and microscopic fibers (Figure 2). In wood cell wall hemicellulose, pectin, and lignin together with cellulose fibrils form a complex bio-composite that offers mechanical support to the tree and protects the tree against environmental factors such as changing climate and pathogens.[9,10]

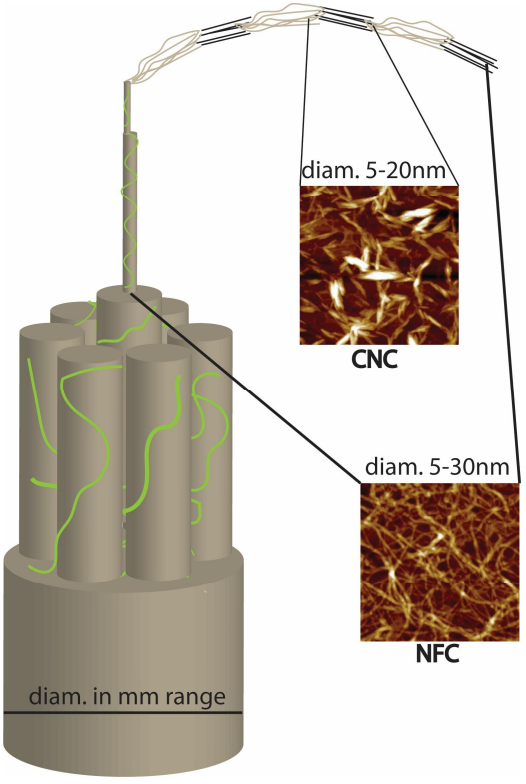


**Figure 2.** A schematic illustration of the hierarchy in macroscopic cellulose fibers. Hemicellulose (shown in green) and lignin are incorporated in the structure between fibers and fibrils. AFM images showing cellulose nanocrystals (CNC) and nanofibrillated cellulose (NFC) point out at which level of hierarchy these materials originate form. The image is not in scale but is only representative.

## 1.1 Nanocellulose

Recently, methods combining chemical, mechanical and enzymatic treatments to disintegrate wood fibers to produce nanocellulose (NC) have been developed.[11–14] Depending on the treatment fibrils with a diameter of 5–30 nm and length in micrometers, or rod-like crystals with a diameter of 5–20 nm and length of tens to hundreds of nanometers to micrometers can be obtained. The fibril/crystal dimensions depend strongly on the treatment and the source but they are always nanosized in at least one dimensions.[3,15–17] The name first adopted for the fibril material from wood source was microfibrillated cellulose (MFC)[18], later the material has been referred to as nanofibrillated cellulose (NFC) or cellulose nanofibrils (CNF).[16,17] The nanosized crystals have previously been called cellulose nanowhiskers or simply whiskers but later a more informative term, cellulose nanocrystals (CNC) has been adopted.[19–21]

### 1.1.1 Nanofibrillated cellulose, NFC

Nanofibrillated cellulose (NFC, Figure 2) can be prepared from wood based pulp material by simultaneous enzymatic and mechanical treatment [22,23] or just mechanical treatment.[24] NFC materials have extraordinary properties due to their nanoscale dimensions. The long and entangled fibrils have high aspect ratio and large surface area exhibiting vast amounts of reactive hydroxyls groups on their surface. [22,25–27] They form an extensively percolating network with low weight percentages. In water NFC forms a gel, which strength is dependent on the fibril concentration. Upon drying NFC forms hierarchical nanoporous aerogels or strong films depending on the drying process.[22,28,29] The mechanical properties of NFC paper films have been reported to be better than ordinary paper and the reinforcing ability in composite materials has also been shown to be superior compared to pulp fibers.[30,31] This is caused by the larger surface area of fibrils exhibiting extensive hydrogen bonding compared to macroscopic fibers. Moreover these films have good gas barrier properties which can be of benefit in many coating and barrier technologies[32]. The aerogels have been shown to have very different properties compared to traditional aerogels due to the long and entangled fibrils forming a hierarchical nanoporous structure.[28] They open up new application areas for the use of aerogels in general. Due to its biocompatibility NFC is also a promising material for biotechnological and pharmaceutical applications.[15,33]

### 1.1.2 Cellulose nanocrystals, CNC

Cellulose nanocrystals (CNC, Figure 2) can be obtained from different sources by hydrolysis with a concentrated mineral acid.[12,20,21,34,35] The treatment hydrolyses the non-crystalline parts of cellulosic materials yielding a dispersion of rod-like crystalline cellulose sticks. These nanosized rods range in size depending on the source

they are obtained from and by the reaction conditions but usually are 5 to 20 nm in width and 0.1 to ~1 µm in length.[20,21,36] CNC has smaller aspect ratio than NFC and in solution does not form as strong gel as NFC due to the lack of extensive percolation of the network.[22,37] Yet the rods are mechanically extremely strong. Experimentally, the Young's modulus of a single crystal of cellulose I is ~134 GPa and the strength in the GPa range.[38] Due to the crystalline rod-like structure, CNC display other interesting properties such as liquid crystallinity.[39]

The mineral acid used in the hydrolysis of cellulose material determines whether the resulting CNC will carry charge on their surface or not.[20] By hydrochloric acid hydrolysis the surface of the crystals will be almost neutral and display almost solely hydroxyl groups. These CNC are usually referred to as HCl-CNC. In this form the CNC are poorly dispersed in water and other solvents due to their tendency to aggregate via strong hydrogen bonding.[13,40] The colloidal stability of HCl-CNC is affected by their concentration and aggregation is more pronounced in higher concentrations.[13] If the hydrolysis is performed with sulfuric acid the resulting CNC will hold sulfate groups on the surface and a net negative charge, referred to as sulfate-CNC. The sulfate-CNC are readily dispersed in water due to their charged surface. They form stable colloidal dispersions in water but can be aggregated with increasing ionic strength.[12,13,20,27] CNC can also be dispersed in some organic polar solvents, such as DMSO.[41]

## 1.2 Applications for nanocellulose materials

There is a growing need for sustainable and more environmentally friendly materials and products in the market to replace plastics and other unsustainable materials. For this cellulose as a material is well suited. Due to its large surface area and high aspect ratio leading to superior mechanical properties compared to macroscopic cellulose fibers and regenerated cellulose, NC could be used in applications where traditional cellulosic products would not be well suited, for example aerogels, composite reinforcement and flexible electronics.[28,42–49] The research done in the field of nanocellulosic materials is growing and new application areas are being explored by the growing knowledge on these very interesting materials. This chapter deals with the application areas where NC could potentially replace other materials, broaden the use of these applications, and show examples on how NC has been used in these fields.

### 1.2.1 Rheology modifiers

Rheology studies the flow and flexure of materials. In many applications it is very useful if the flow of matter can be altered during processing. Altered flow properties can for example allow broader applicability for existing materials. Gels with percolating networks can be used to modify the rheological properties of other materials when mixed together. The need to modify how matter stands applied force is often useful in such cases as in paint and lacquer applications, in cosmetic applications,

in polymer processing, and in food technology. In many applications it is useful if the rheological properties of the material can be tuned to fit certain needs for example in processing; e.g. to ease the spreading of paint to wall or melt processing or extrusion of a polymer, or simply adding strength to the material in wet state in different environmental conditions.[50–52] On the other hand the rheological properties of a material determine how it can be used in specific applications, such as hydrogels, reinforcing phase in composites, thin film applications or emulsion and foam stabilization.

NFC forms a strong gel in aqueous media in broad range of concentrations in contrast to CNC dispersions which form gels only in very high concentrations. Pääkkö et al. have studied NFC (prepared from soft wood sulfate pulp by mechanical and enzymatic means) rheological properties. They showed that these materials display typical ideal gel behavior, i.e. elasticity, that is the storage modulus, G', is much greater than the loss modulus, G'', and G' is independent of frequency in low solid content dispersions (0.125 % w/w) as well as in high (5.9 % w/w).[22] Shafiei-Sabet et al. on the other hand have shown that CNC with net negative charge behave like viscous fluids in low concentrations (< 10 % w/w), the behavior is then gradually changed to weak gels (~12 % w/w) and to stiff gels with G' (~$10^2$ Pa) > G''.[53] The strength of the gel or dispersion, i.e. G'-values, were dependent on the concentration in both cases. The modulus of NFC was noted to be 2-orders of magnitude higher for NFC dispersions compared to CNC dispersions, which showed that NFC forms a strongly percolated entangled network compared to CNC. The NFC dispersion showed shear thinning behavior, which is typical for such gels. For CNC dispersions, at lower concentrations (1–4 % w/w), different states during the shear thinning process were observed; a transition from isotropic to liquid crystal (LC) phase, the destruction of LC phase and the orientation of individual CNC rods along the shear force. For CNC at higher concentrations (> 4 % w/w), the transitions from alignment of LC to aligned LC phase to destruction of LC phase and formation of gel were seen. The higher shear viscosity of NFC compared to CNC is due to the entangled network that prevents the individual elements from flowing under increasing shear in contrast to CNC dispersions, where the elements are interconnected less extensively and can flow upon increased shear.

The presence of hemicellulose in the NFC material and its effect on the viscosity was also studied[22]. It was noted that the net negative charge of the material (44.2 µeq/g) was solely caused by the presence of hemicellulose. The viscosity of the dispersion was increased by the lowering of pH, i.e. the amount of negative charge in the material was reduced. This reduction of negative charge decreased the repulsion between the fibrils and allowed more interfibrillar interactions that lead to increase in viscosity. The opposite was seen upon increase of pH; more negative charge on fibrils caused more charge and more repulsion between fibrils, this in turn lead to less interfibrillar interactions and lower viscosity.[22] The same observation of effect of charge was seen with CNC when two preparations with lower and higher net negative charge were compared.[53] The flow properties of HCl-CNC and sulfate-CNC have been studied by Araki et al. [13]. In their study they conclude that both CNC show shear thinning properties yet HCl-CNC more strongly. The time dependent

viscosity measurements showed that the viscosity of sulfate-CNC dispersions was not dependent on time and was a stable colloid throughout the experiment. On the other hand the viscosity of HCl-CNC was time dependent; high concentrations (>5 $gL^{-1}$) showed thixotropic behavior (decrease in viscosity) and low concentrations (<3 $gL^{-1}$) anti-thixotropic behavior (a slight increase in viscosity was seen). The structurally very similar materials showed remarkably different flow properties and thus were concluded to be useful with different applications in respect to each other.

These investigations highlight the importance of the rheological properties of NFC and CNC in relation to many applications. The dependence of the viscosity and strength of the gel or dispersion on concentration and the shear thinning behavior can be especially interesting for many applications where ability to tune material properties and change behavior according to processing are desired, yet where it is important that gel properties and the entangled network structure are preserved. Also the fact that CNC undergo phase transitions during shearing process is relevant in many cases. Due to the different rheological behavior of CNC dispersions compared to NFC, i.e. lower viscosity, weaker dispersions/gels, and liquid crystallinity, the applications for them would be somewhat different. Although both materials show promise in strengthening composites and hydrogels.[54]

### 1.2.2 Composites

Composites are materials that combine two usually very different materials together in a way that the mechanical properties of the composite are better compared to the materials that it is made up of alone. Composites usually contain a softer continuous phase and a stiffer or harder reinforcing phase. The materials in composites do not dissolve into each other but are separate and can be distinguished from one another.[55–57]

Practically all natural materials are composites. These materials are in many perspectives superior in their properties compared to manmade ones. For example the mechanical properties of natural ceramics (e.g. bone, shell, and dentine) combine stiffness and toughness witch is very hard to achieve in synthetic analogs. Their moduli are lower than those of synthetic ones, but their tensile strengths are similar and their toughness is much greater. Natural polymers (e.g. cellulose, chitin, collagen, and silk) have much larger moduli and tensile strengths than engineered polymers, excluding Kevlar fibers, which have highly oriented molecular structure, as do natural polymer fibers, and covalent bonding.[58–60]

Natural composites illustrate very well the effect of structure within the composite to the overall mechanical properties of the material. An example is the differences in mechanical properties of tendons, ligaments, skin, cartilage and bone. They are all composed mainly of two components; collagen and elastin. The distinction of the materials and thus their very different properties lie in the fraction and architecture of each component.[58] All natural composites have hierarchical structure from nanoscale to macro scale to mesoscale. At the sub-nanoscale the self-assembly of atoms and molecules will drive the formation of nanoscale structures that in turn will

self-assemble and cause large well defined structures to form and these in turn induce the next scale structures to form. This is called the bottom-up method of building structures and this is the way materials and structures are built in nature.[57,59,61] The hierarchical assembly of natural composites adds fracture toughness and allows better energy dissipation within the material when outside force is applied.[57,61] Manmade composites are blends of two materials and their properties are mostly dictated by the manufacturing process.[55] Recently attempts to mimic nature's way of bottom-up approach have been taken into practice in making engineered composites.[56]

Two features are important when considering a composite material: 1) The interface between the two materials should interact with each other in order for the material to transfer applied stress and load during impact. 2) The reinforcing material should be well dispersed into the soft phase otherwise no reinforcement or only local reinforcement occurs which does not allow reinforcement throughout the whole material.

The strength of a composite can be studied by the relation between stress and strain. The stress is the applied force on an object divided by the area of where it is applied to, and the strain is the resulting deformation of the sample. Typically if the applied stress is small the material will undergo elastic deformation, i.e. it can recover to its original shape when stress is released. In this elastic region the strain is linearly proportional to the stress. When applied stress becomes higher the response of the material becomes non-linear but can still be elastic. Above a critical point (yield point) the response of the material becomes plastic and the material will not recover to its original shape after stress is released. The strain-to-failure will occur when the material ruptures. The tensile modulus, E, or Young's modulus is determined in the linear-elastic region of tensile tests and is the ratio of tensile stress and tensile strain (elongation).[58]

For composite materials both NFC and CNC could be interesting materials. NFC can be used as a percolating network of strong interconnected fibrils which is embedded in a softer polymeric matrix. In the same way, CNC can be used to reinforce a softer matrix material to strengthen the system. NFC can also been used as a soft, yet strong, connecting material together with tough and strong materials such as graphene to mimic nacre architecture.[19,25,48]

Abraham et al. present an example of a natural rubber latex (NR) – NFC –composite (NR/NFC-composite) where the NR matrix has been reinforced with NFC obtained from jute fibers by steam explosion.[62] The NR/NFC-composite was either cross-linked or not and the biodegradability, morphology, crystallinity and mechanical properties were studied. The biodegradability of NR/NFC-composites was confirmed and the more NFC the materials contained the better they were degraded. Cross-linking of the composite hindered the biodegradability, yet the cross-linked composites showed superior mechanical, thermal and barrier properties compared to non-cross-linked composites. They propose a mechanism for the cross-linking and show that due to the cross-linking reaction, the crystallinity of NFC is totally lost. So in the sense reinforcement of the composite does not come from crystalline cellulose but well dispersed amorphous cellulose fibrils or chains interacting with and

cross-linked to the NR-matrix. In the non-cross-linked NR/NFC-composite the crystallinity of NFC is preserved and the composites compared to non-cross-linked NR alone show higher Young's modulus and tensile strength, yet the ability of the composite to elongate is hindered compared to NR. This shows that NFC fibrils can reinforce the NR matrix.

Another example of NFC used as a reinforcing material is the work of Fernandes and co-workers where they used NFC to reinforce chitosan to prepare transparent, flexible, mechanically and thermo-mechanically better films compared to neat chitosan films.[63] The preparation was done by simple mixing of solution of chitosan (high or low molecular weight chitosan in acidic solution, or their water-soluble derivatives) with different amounts of NFC (maximum of 60% NFC in the film). The mechanical properties were improved drastically by the addition of NFC into the matrix (improvement of Young's modulus of up to 150% with the water-soluble high molecular weight chitosan and 320% with the water-soluble low molecular weight chitosan). The thermo-mechanical properties and the thermal stability of the composites were also better than those of the neat chitosan.

NFC can also be used as the "soft" or "continuous" phase of a composite and be reinforced with a harder material. An example of this is presented in the work of Aulin et al. where nanoclay particles are used to reinforce NFC matrix resulting in optically transparent, flexible biohybrid films with stiff and strong mechanical properties, and better oxygen barrier properties compared to the NFC alone.[44] In this work, interaction between the NFC matrix and nanoclay particles is enhanced by surface modification of NFC so that the fibrils carry a net negative charge.

Capadona et al. have shown in their work how CNC reinforced polymer matrix results in a stimuli-responsive composite material inspired by the sea cucumber dermis.[64] They showed that CNC from tunicate reinforce the polymer matrix and upon a stimuli (depending on the polymer used; for example chemical or thermal) the mechanical properties are changed. They show that the interaction of the chemical stimuli with the CNC in the matrix causes the disturbance of the percolating network of CNC and cause the lowering of mechanical properties. They show that these materials could be useful in biomedical applications serving as adaptive substrates for intracortical microelectrodes.

### 1.2.3 Porous scaffolds

Hydrogels and aerogels, both porous materials to the nanometre scale, are promising for making stimuli responsive materials, to embed and transfer functional moieties, for architectural scaffolds, for microelectronics, acoustics, and optics among other applications.[65–68]

#### 1.2.3.1 Hydrogels

Hydrogels are hydrophilic polymeric networks that can retain large amounts of water and still maintain their three dimensional structure. The hydrogel network is either

chemically (covalent bonds) or physically (van der Waals, charge or hydrophobic interactions) cross-linked and it can be stimuli responsive (heat, light, chemical etc.) in adsorbing and desorbing water.[67] There are numerous examples on hydrogel usage in for example bio-medical applications such as tissue engineering, diagnostics, and drug delivery [69–73], in pharmaceutical and cosmetic applications [74], as adsorbent materials in diapers and other everyday items, and in fillers and paints.[67] Traditional hydrogels are not strong in their mechanical properties due to the large amount of water they hold, and thus do not allow applicability in areas where load bearing is needed.[67,68]

NFC forms hydrogels in low weight to volume ratio when it is produced. The gels are mechanically strong and the strength is dependent to their solid content. The NFC network is physically cross-linked by interfibrillar hydrogen bonding. The fact that NFC is fully biological and biodegradable also offers a possibility to replace polymer-based hydrogels in existing applications.[22]

NFC hydrogels offer a good platform for biotechnological applications such as tissue engineering and cell culture where hydrogels are often used, due to its three dimensional well hydrated porous scaffold with desirable and tunable structure, low toxicity and biocompatibility. This has been demonstrated by Bhattacharya and coworkers.[73] In their work they show that the hydrogel of native NFC from wood source serves as a three dimensional extracellular matrix mimic for liver cell growth and differentiation. No cytotoxicity was observed, and the rheological properties of NFC were noted to be beneficial for processing of the material into three dimensional cell cultures; specifically, it was injectable and allowed mixing of cells into the gel structure, yet provided the mechanical strength that was needed for the support of cell growth and differentiation.

CNC can be used to reinforce stimuli responsive hydrogels to give better mechanical properties compared to the hydrogel alone.[54] McKee et al. used mechanically strong CNC to reinforce thermo-responsive yet mechanically weak methylcellulose (MC) to yield an all-cellulose thermo-reversible and tunable nanocellulose-based hydrogel. The mechanical properties were improved by the growing concentration of CNC in the matrix while the MC concentration remained constant throughout the study. At low temperatures (20 °C) and low CNC concentrations the hydrogels behaved as viscous dispersions, upon increasing CNC concentration weak gels were obtained. At higher temperatures (60 °C) the hydrogels behaved as distinct gels. McKee and colleagues conclude that they show a facile and scalable method for preparation of fully cellulose containing nanocomposite hydrogels with a broad window of viscoelastic properties that can be tuned simply by choosing CNC concentration and temperature according to the need of the application.

#### 1.2.3.2 Aerogels

Aerogels are ultra-light weight porous solid materials traditionally made of inorganic silicon dioxide.[65,66,75] They can also be manufactured from other materials such as

carbon, carbon nanotubes and graphene, or other organic materials, which can also be pyrolyzed to carbon.[76–81] The porosity of an aerogel is very high (usually over 75 %) and they are usually transparent and very brittle due to this reason.[75] To enhance silicon based aerogels mechanical properties organic-inorganic silicon based aerogels have also been prepared. Aerogels have an open-pore structure and they have large surface area due to their structure. The traditional $SiO_2$-based aerogels have a structure that resembles string of pearls (the particle size being ~4nm) joint together from here and there so that the voids in between the strings are in average ~30 nm (Figure 3). Aerogels are usually made from solvent swollen wet gels by supercritical drying process. The key in preparing the aerogel is that the percolating network of solid material is not lost (prevention of collapse and shrinkage of network is essential) during the drying process.[65,66,75]

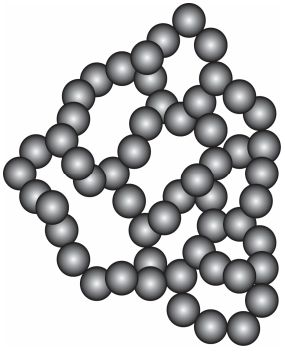

**Figure 3.** A schematic illustration of a traditional aerogel based on silicon. The particles are connected to each other like pearl neckless, structures are three dimensional and the voids are typically ~30 nm in size. Adapted from [75].

Aerogels have applications in many fields ranging from chemistry to material science to physics; they are used in Cerenkov detectors, they can be used as insulators, in passive solar energy usage, as catalytic substrates, in acoustics, and as filters to name a few.[75]

NFC forms a nanoporous hydrogel as it is prepared. This hydrogel can be used to prepare light weight flexible aerogels with very different properties compared to the $SiO_2$-based and carbon based aerogels.[28,47,49,82,83] Cellulose-based aerogels have been prepared previously but their emphasis has been on using regenerated cellulose derivatives (cellulose II) or cellulose-starch blends.[84–88] Cellulose II is mechanically weaker than the native cellulose I so NFC offers a feasible route for mechanically strong, biodegradable, bio-based platform for aerogels.

There is a growing number of examples on NFC aerogels used to prepare for example functional materials.[45,89–92] One of the first to show the preparation and applicability of NFC aerogels were Pääkkö and co-workers.[28] They showed that NFC hydrogels without chemical cross-linking can be used to prepare flexible and

mechanically durable aerogels in vacuum oven by freeze-drying. Due to the entanglement of the fibrils in the network no cross-linker was needed and the cross-linking of the aerogel was caused by physical intermolecular interactions (hydrogen bonding) of fibrils. In contrast to inorganic and organo-modified silica aerogels, NFC aerogels showed an order of magnitude higher strain and had maximum compression strength of ~200 kPa. These results highlight the fact that NFC aerogels are not brittle as traditional aerogels. Pääkkö et al. also show that these aerogels can be used to prepare conducting aerogels simply by immersing the aerogel to a solution of conducting polymer with surfactant. The polymer used in their work was polyaniline doped with dodecyl benzene sulfonic acid.

Jin et al. show how NFC aerogel can be used to create superhydrophobic and superoleophobic materials and show their applicability as cargo carriers on water and on oil.[49] The material was shown to be able to carry a load much larger than its own weight. They show that these materials mimic the water striders in their ability to form a layer of air between the solid material and water (a plastron layer). This was also shown to occur in oil. The material showed reduces viscous drag when it was in contact with oil, which originates from its ability to create the plastron layer. The material was also shown to selectively permeate gases by not fluids.

These examples show that NFC aerogels can be applied in very different ways than the traditional aerogels made of inorganic silica, paving the way towards green and bio-based aerogels.

### 1.2.4 Stabilization of surfaces and interfaces in foams and emulsions

In many foam and emulsion applications, especially in food, pharmaceutical, and cosmetic industries, the stabilization of surfaces and interfaces is a key issues for product development.[93,94] Another way to improve foam and emulsion stability is to modify the properties, for example viscosity, of the continuous face. Traditionally foams and emulsions can be formed and stabilized by the presence of amphiphilic molecules such as detergents. These molecules are considerably small in size and contain a longer hydrophobic moiety and a small hydrophilic moiety, such as sodium dodecyl sulfate (SDS), which is commonly used in everyday items like toothpaste. Detergents self-assemble to cover gas or oil droplets in water environment so that the hydrophobic side of the molecule is facing the gas/oil (i.e. the hydrophobic material) face and hydrophilic facing water, or the hydrophilic material (Figure 4A). They lower the surface or interfacial energy and thus allow the formation of foams and emulsions and keep the formed gas bubbles or oil droplets apart. Traditional detergents do not stabilize the foams and emulsions very well allowing the coalescence of oil droplets or gas bubbles during time.[93,94] In foams this is known as Oswald ripening where gas flows from smaller bubbles to larger bubbles causing the loss of foam structure.[94] More effective stabilization of surfaces and interfaces is gained by the use of polymers.[95] Once adsorbed to the surface or interface they are more efficient in keeping the interfaces apart. [93,95]

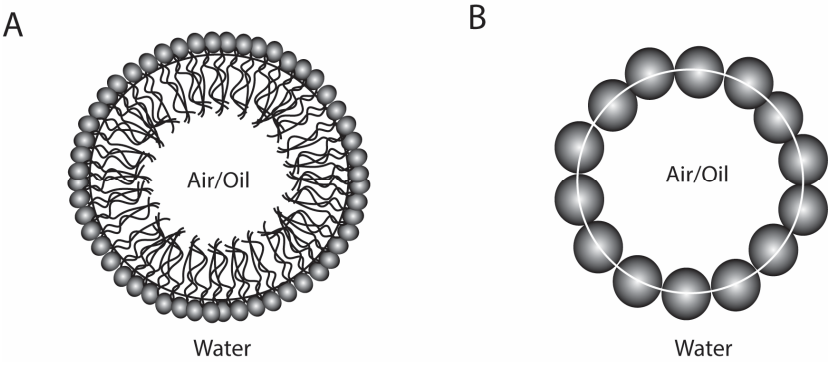


**Figure 4.** A schematic illustration of A) detergent, such as SDS, stabilized emulsion/foam, and B) a solid-stabilized emulsion (Pickering emulsion)/foam.

Along traditional emulsifiers and foaming agents, solid stabilizers of nanoscale size have been developed. These solid particles self-assemble on surfaces and interfaces and produce more stable foams and emulsions. Solid-stabilized emulsions are also called Pickering emulsions. The solid particles assemble on the interface due to their tendency of being partially wetted by water and partially by the hydrophobic matter (Figure 4B). The same principal drives them to gas-water interfaces.[96,97] These solid particles are also called Janus particles due to their two-phased nature.[98–100] Janus particles exist in nature as well, for example the protein amphiphile, hydrophobin, from fungi is well known to lower the surface tension of water by self-assembling onto the air-water surface and allowing the hyphae to penetrate through the surface. [101,102] They also readily assemble to any hydrophobic material, liquid or solid.[101,103–105]

Nanocellulose having nanoscale dimensions could be a useful bio-based solid colloidal material for the stabilization of emulsions and foams.[106–112] It is known that in the crystalline cellulose parts of cellulose I the cellulose chains are arranged in such a fashion that the different faces of the crystallites have different properties; two of them are more hydrophobic while the other two are clearly hydrophilic.[113,114] This is a useful feature in NC that could aid its assembly at interfaces and potentially be used as a stabilizer in emulsions and foams. Also the fact that NC is bio-based and bio-degradable adds interest in using them instead of polymers and non-biological solid particles especially in the fields of food, pharma, and medical technologies.

In literature many times the phrase “foam” is used instead of “aerogel”, which the material actually is. I will here make a difference in that an aerogel is a material that has been prepared by the exchange of liquid to gas as described earlier. By foam is meant a structure where gas in one way or another has been trapped in a matrix containing liquid and possibly solid material. The foam can be dried after preparation to yield solid foam or dry foam.

In our work in Publication (I) we saw that both CNC and NFC assemble onto the oil droplet interfaces creating oil-in-water emulsions and that especially NFC had a significant effect on the emulsion stability. In addition to the assembly at the interface, NFC also changed the viscosity of the continuous phase (water) of the emulsions and thereby hindered the movement of the oil droplets within the matrix thus stabilizing the emulsion further compared to CNC.

Other examples of emulsion stabilization by NC materials are presented by Sèbe and colleagues[112], who used CNC, and Ougiya and colleagues[115], who used bacterial cellulose (BC). Cervin et al. have used NFC to stabilize foams[108].

In the first example, Sèbe et al. created Pickering emulsions of oil-in-water type with surface modified CNC. They had two different surface modifications and three different non-polar to slightly polar phases that were used to create the emulsions. The surface modifications were vinyl acetate and vinyl cinnamate, and they saw that the modification had an effect on the emulsion stability. They speculate that the vinyl cinnamate modified CNC emulsions could be useful in creating photo responsive emulsion systems. They also propose that the emulsion stabilization of such a system based on polymerizable monomers could be applied to polymerization in dispersion media.[112]

Ougiya et al. investigated BC compared to other more macroscopic cellulose materials (microcrystalline cellulose, MCC, microfibrillated cellulose, MFC), a natural polymer (xanthan gum), and a small surfactant (sorbitan monolaurate). They saw that BC stabilized the emulsions better than the rest of the cellulosic materials. This was due to its fine nanoscale size in comparison to the coarser MCC and MFC. It was also noted that the BC stabilized emulsions were stable against pH change, temperature change and NaCl concentration which neither the polymeric material nor the surfactant were. They saw that in addition to creating a mechanical barrier on the oil droplet surface, BC fibrils were observed in the continuous phase of the emulsion forming a scaffold for the oil droplets. This scaffold structure in their opinion contributed to the stability of the emulsion. This seems to be the same observation that we had with NFC stabilized emulsions, specifically that the fibrils in the continuous phase, in addition to the mechanical barrier on the droplet surface, serves to stabilize emulsions.[115]

Cervin et al. have shown that carboxymethyl modified NFC can be used together with a polymeric substance to stabilize foams and that these foams can be dried to solid foams without the collapse of the foam structure. The lifetime of the aqueous foams was significantly prolonged compared to foams that were not stabilized with NFC. The mechanical properties of the solid foams were measured and noted that the Young's modulus of compression was better than that of freeze-dried cellulose aerogels but lower than that of polystyrene foams. The structure of the aqueous foams was investigated by different microscopy methods. They saw that NFC assembled on the air bubble surfaces. They speculate that this assembly of the polymer coated NFC on the surface inhibits the diffusion of air from smaller bubble to larger bubbles and thus inhibits the coalescence of the bubbles and causes the stability of the foam.[108]

### 1.2.5 Thin films

Thin films are used in many different applications including electronics, coating and pharma technologies, biosensors, as well as employed to understand fundamental material interactions.[40,116–122] These films are often prepared on solid supports by spin-coating[123], solvent casting[124], chemical vapor deposition (CVD)[125,126], atomic layer deposition (ALD)[127] or prepared on a Langmuir trough by self-assembly of molecules onto air-water/oil-water interface and then lifted on solid supports by either Langmuir-Blodgett (LB) or Langmuir-Schafer (LS) -method[128–130]. Molecules with a self-assembling tendency can be adsorbed directly onto solid supports from bulk[101,104]. These different methods can be used to prepare thin films with monomolecular layers, or multiple layers of altering (or the same) materials referred to as layer-by-layer deposition (LbL)[131]. LbL allows a controlled architecture of alternating layers of different materials to be present in a film. Polymers, metals and nanomaterials are used for preparation of thin films for their desirable and tunable properties, which include conductivity, porosity, hydrophobicity, and plasmon resonance. Self-assembly is a feature that is, in most materials that form thin films, a key for the preparation and structure of the resulting film. Static self-assembly occurs when molecules assemble to reach a thermodynamic equilibrium or energetic minimum at certain conditions.[132]

CNC and NFC readily form thin films upon for example spin-coating, casting, LS-, and LB-techniques[32,133–135]. NFC thin films have been used as model films to study the interactions of native cellulose with several other molecules, such as hemicelluloses and cellulases[136,137]. NFC has also been used to prepare flexible, transparent organic light emitting diodes (OLED)[46]. The multilayers of NFC and polymeric materials have also been prepared and their mechanical and adhesive properties have been studied[138,139]. CNC has been used to create thin films with birefringence properties due to their rod-like structure[140]. These materials would offer an environmentally friendly approach for electronic and other thin film applications.

In Publication (II) of this thesis, we have investigated the use of NFC to create bioactive thin films that could be used in diagnostic applications but also in other applications where biomolecule immobilization is required, such as enzymatic pretreatment in paper making and other industrial scale processes that take advantage of enzyme reactions. In our work, we used NFC to take the immobilization chemistry from solid surface (2D) to bulk (3D), by attaching the biomolecules to NFC fibrils in aqueous dispersions. After the coupling the NFC ability to create films upon spin-coating was exploited in creating porous thin films with bioactivity. The benefit of NFC in this case was also the fact that multiple layers could be spin-coated and due to the porosity of the films bioactivity was seen throughout the whole film.

Cranston et al. used LbL deposition of NFC and polyethylenimin (PEI) to create thin films of different thicknesses of alternating NFC and PEI.[139] The elastic properties of the films were investigated by strain-induced elastic buckling instability for mechanical measurement (SIEBIMM) technique. This technique allows the determination of Young's modulus of thin films with thickness down to ~40 nm. In their

work Cranston and co-workers show that NFC thin films and SIEBIMM are useful tools to investigate how the properties of the thin films change with humidity and thickness, provide in-depth information on the fibril-fibril and fibril-polymer interactions and show that SIEBIMM is an applicable technique to large aspect ratio colloidal particles. They show that humidity causes a significant decrease in the mechanical properties and highlight that in order to achieve strong reinforcement by NFC extensive cellulose-cellulose interactions are needed and water adsorption has to be minimized. They predict that the technique will be useful to study different combinations of NFC (modified with anionic or cationic groups), lignin, and hemicelluloses to mimic and understand the interactions of thin lamellae in the fiber wall but can also be applied generally to determine moduli of films containing nanofibers, nanocrystals, dissolved cellulose, and other biomimetic thin films.

## 1.3 Modification of nanocellulose surface properties

The properties discussed above make NFC and CNC very promising materials for rheology modifiers, mechanical reinforcement, as templates for smart materials, in barrier technologies, flexible electronics, and many biotechnological and biomedical applications.

CNC prepared by HCl-hydrolysis has a very poor colloidal stability. This is due to lack of repulsive forces between the crystallites and thus the suspensions are very unstable. The use of $H_2SO_4$ in the hydrolysis adds enough negatively charged groups (sulfate) on the crystal surface to create repulsion between the individual rods and thus yields a more stable dispersions in aqueous media. If one wants to embed them in other materials such as hydrophobic polymers further surface modification is needed for sufficient interaction to take place between the two materials at the interface.

The same need for surface modification applies for NFC yet it is more stable in aqueous media after preparation than unmodified CNC. In NFC materials derived from wood source this is most probably due to the hemicelluloses present in the material acting as a colloidal stabilizer and preventing aggregation. NFC is also less crystalline and contains substantial amount of unordered amorphous parts in the fibrils that do not aggregate as strongly as crystalline regions. Nevertheless NFC is very sensitive to processing and tends to flocculate or aggregate upon different treatments such as solvent exchange, application of mechanical force etc. (III). Blending unmodified NFC or CNC together with other materials rarely works out due to the lack of interaction of the fibril surface with the other materials. In other words, the cellulose crystal and fibril surfaces are passive and do not readily interact with other materials.

To overcome these issues NFC and CNC surfaces need to be modified for their efficient use in most applications. The modification of NFC and CNC can be grouped to chemical and physical modifications as with any colloidal particles. Chemical modifications involve covalent bonding and the chemistries are usually done in solvent media. They can involve chemicals that are hazardous, such as azides or metal

catalyst, and the reaction conditions can be harsh, including extreme pH, high temperature, and inert gas environment. Physical modifications are generally done in less harsh conditions than chemical; they involve adsorption of molecules on to the surface via weaker chemical interactions than covalent bonding i.e. electrostatic interactions, hydrophobic interactions, or van der Waals interactions.

In the following sections the different modifications and their use in functionalization of NFC and CNC will be discussed.

### 1.3.1 Chemical covalent modification of nanocellulose surface

The different chemical modification of NFC and CNC can be divided roughly into three major groups; negatively charged, positively charged, and hydrophobic NC. The degree of modification will affect the materials properties. In the case of CNC the amount of negative groups on the surface will determine how well the individual rods are dispersed and in what concentrations they will behave as gels or liquid crystals.[37,53] In the case of NFC chemical modification can reduce the amount of hemicellulose and affect the colloidal stability (II, epoxy modified NFC) or it can alter the pH sensitivity of the material [141]. The idea in NFC and CNC modification is to sustain the strong cellulose I crystal structure and to modify only the very surface of the fibril or crystal. Thus, the modification conditions need to be kept mild enough in order not to swell and subsequently dissolve the cellulose.[15] Also flocculation needs to be avoided and a good dispersion of fibrils and crystals during modification is important so that the product is evenly modified.

#### 1.3.1.1 Anionic functionalization

As described earlier, CNC can be produced in such a way that the surface of the product will hold a net negative charge. This is simply done by choosing the acid for the hydrolysis reaction accordingly; sulfuric acid will derivate the resulting CNC surface with sulfate groups and hydrochloric acid will not derivate the CNC surface.[13] The sulfate-CNCs can readily interact with positively charged molecules[142], can be dispersed in water and some polar solvents[41], and can be used for further modification or functionalization[143]. The HCl-CNC can also be modified after production either to contain sulfate groups (treated with sulfuric acid) or to contain carboxylic acid groups[144]. The latter method is mostly applied to NFC materials and will be described in the following paragraph[145].

To produce negatively charged NFC with a very fine and even size distribution, a method called TEMPO (2,2,6,6,-tetramethylpiperidine-1-oxyl) -catalyzed oxidation has been developed.[146] TEMPO-oxidation of cellulosic material is solely specific to the C-6 hydroxyl (primary alcohols). The specificity and well controlled conditions of the reaction allow the reaction to take place only on the primary alcohols exposed on the fibril surface. The modification leads to fibrils where every second AGU on the fibril surface carries a modified C-6 hydroxyl. Due to the specific nature and well controlled reaction conditions TEMPO-oxidation produces cellulose fibrils with a

very uniform diameter depending on the source they originate from.[147–149] The properties of TEMPO-oxidized NFC (TEMPO-NFC) are very different from the original non-modified NFC first due to the high density of negative charge on the fibril surface and second due to the fibril dimensions being smaller and more homogeneous.[145,147] The size of TEMPO-NFC fibrils and their fine dispersions in water allow fully transparent hydrogels with high viscosity in comparably small solid content and they form transparent films with high oxygen barrier properties upon drying[32,145]. The wettability of TEMPO-NFC is very different from that of neat NFC. A dry TEMPO-NFC film adsorbs water almost immediately but this can be reduced by surface hydrophobization commonly used in papermaking. Also the thermal degradation of TEMPO-NFC is altered and it is lower than that of unmodified fibrils. The thermal expansion on the other hand is very low, much lower than that of glass.[32] This feature together with the high transparency makes TEMPO-NFC a potential substrate for flexible electronics. The large surface area, high aspect ratio and high charge density offer an interesting starting material also for further modification and functionalization, which has been investigated for example in Publication II of this thesis by conjugation of enzymes to TEMPO-NFC.

#### 1.3.1.2 Cationic functionalization

For interactions with negatively charge materials, such as kaolin clay[150] and oxidized graphene[151], for further modifications with functional groups, such as proteins (II), or to add strength and antibacterial properties to materials[152,153], cationic groups have been introduced on NFC surface. Different routes to prepare cationic NFC have been reported in literature[141,150,152–154]. These reactions rarely alter the rheological properties of NFC dispersions or the fibril morphology too dramatically, as compared to for example TEMPO-mediated oxidation, but rather introduce reactive groups on the fibril surface with lower degree of substitution.

In Publication (II) we show one route to alter the surface of NFC by introduction of amine groups in order to further modify NFC with bioactive moieties, i.e. enzymes. The synthesis was performed via silylation reaction originally tailored for dissolving pulp. The amount of amine groups in the modified NFC was calculated to be 1.24 mmol $g^{-1}$ cellulose and the degree of surface substitution for the material was ~0.2. The surface substitution was possible to estimate since x-ray photoelectron spectroscopy (XPS) was used to analyze the surface silicon and nitrogen content. In contrast many times the total substitution level of the material is reported and then assumed that all reactive groups lie on the fibril surface. In the latter case the fibril dimensions will affect the surface substitution degree and vary accordingly.

#### 1.3.1.3 Hydrophobic functionalization

Hydrophobicity of NFC and CNC materials is often desired for interactions with polymers that are hydrophobic. This is usually done by polymerization reactions either

from or onto fibril and crystal surface. There are numerous examples of these reactions and they are well reviewed.[15,155,156] In some cases hydrophobicity in combination with nanoscale hierarchical structure of the material can lead to super hydrophobicity of the resulting material[157]. Another reason for hydrophobizaton of NFC or CNC would be that some of these groups are easily reacted further. An example of such modification is the epoxy moiety. Epoxy groups are well known to react with amine groups and can be used to cross-link materials (epoxy glues) or for example covalently immobilize proteins onto solid supports[158–160].

In Publication (II) of this thesis we made and examined the use of epoxy modified NFC in protein conjugation and immobilization. The epoxy containing NFC was prepared through an allylation step described earlier by Huijbrechts et al. for starch. The amount of epoxy groups for the final NFC material was analysed to be 0.64 mmol $g^{-1}$ per glucose and calculated to correspond to a degree of substitution of 0.061. This is a total degree of substitution and the surface substitution would be higher assuming all epoxy groups were on the fibril surface. This assumption is justified since the fibrillar structure of the NFC material was sustained and not destroyed by the reaction conditions. The surface substitution in this case is very hard to estimate due to the broad size distribution of the fibrils in the material.

### 1.3.2 Physical adsorption of molecules onto nanocellulose surface

For NFC and CNC containing charged groups on their surface the adsorption of other molecules can be performed using charge interactions. This can be used to decorate the fibrils or crystals with functional groups or to yield better interaction with another material in composites. CNC and NFC can also be readily modified by adsorption of other carbohydrate containing molecules such as hemicelluloses and branched polymers containing sugar moieties[137,161–163]. In nature there are also proteins that specifically adsorb to cellulose surface. These are called cellulose binding modules (CBM, or cellulose binding domains, CBD)[1], and they are produced by organisms that can degrade cellulose, for example some fungi and some bacteria.[164,165]

#### 1.3.2.1 Adsorption of hemicelluloses to cellulose

It has been observed that other natural carbohydrate containing polymers, i.e. hemicelluloses, of different sizes interact with cellulose surface and adsorb readily[26,137,162,163,166–168]. The adsorption is dependent on their size and branching; the larger they are the more they adsorb and the more branched they are the less they adsorb. For example studies on xyloglucan adsorption have shown that polymers

[1] In the first publication (I) the term CBD was used. In this thesis and the fourth manuscript (IV) the term CBM is used.

smaller than 12 sugar units do not adsorb on cellulose surface. For xylan the critical length has been shown to be 15 units.[163,169]

NFC from wood source has always residual hemicelluloses; from soft wood source galactoglucomannan, and from hard wood source glucuronoxylan [170,171]. The hemicellulose in pulp has been shown to aid fibrillation the process[170]. The drying of pulp has also a negative effect on the fibrillation process; the fibrils coalesce irreversibly by the formation of strong hydrogen bonding and due to the contamination of the fiber surface, it is made passive or less reactive. It has been shown that the more hemicellulose is present in the pulp the less it suffers from the effects of drying.[170,172–174] In this regard, it can be considered that NFC from wood source has already a natural modification on the fibril surface by the physical adsorption of hemicellulose. This surface modification of NFC has an impact on the preparation and handling of the material but also on the mechanical properties of the products prepared from it. This was seen in the third publication of this thesis (III) where the role of xylan in hard wood NFC matrixes was studied by specifically removing xylan by xylanase enzyme. It acts as a colloidal stabilizer of the fibrils[22] but it also affects the chemical modification of NFC material. It is more reactive than cellulose and the conditions in the reactions solubilize hemicelluloses making it more accessible for the reagents than cellulose. Thus hemicelluloses usually lower the reaction yields. This was seen for example in the second publication of this thesis (II), when NFC was modified with epoxy functionalization.

#### 1.3.2.2 Specific adsorption of cellulose binding module, CBM, on nanocellulose

In nature many organisms ranging from fungi to bacteria produce proteins or peptides that have the ability to bind specifically to crystalline cellulose. In the filamentous fungus, *Trichoderma reesei*, some of the cellulose degrading enzymes, cellulases, have a CBM, which are members of the family 1.[164]

The CBMs of the two major cellulases of *T. reesei*, cellobiohydrolase I (previously named CBHI, later Cel7A) and cellobiohydrolases II (previously named CBHII, later Cel6A) have been studied extensively.[2] Recent and past reviews offer a good overview on what their role for the cellulase action is proposed to be[164,175]. The enzymes have two functional domains; a core enzyme with the catalytic activity, referred to as catalytic domain (CD) and the CBM which is attached to the core enzyme by a relatively long linker that is O-glycosylated (Figure 5A).[176,177] The fungal family 1 CBM proteins are small peptides of ~3 kDa in size. The amino acids that are crucial for the binding have been identified. For the CBM-CEL7A there are three tyrosine residues (Y5, Y31, and Y32) on the binding face which are critical (Figure 5B) and for CBM-CEL6A there are two tyrosine residues (Y33 and Y34), and a tryptophan

[2] In Publication I the nomenclature CBHI and CBHII have been used instead of Cel7A and Cel6A. In this thesis and in Publication IV the nomenclature Cel7A and Cel6A has been used.

residue (W7) responsible for the binding.[178,179] By mutating these residues the binding properties of the CBMs are altered[180]. The affinity of the CBMs has been previously studied with substrates such as BMCC, tunicin, Avicel and chitin. It has been shown that the binding affinities differ on different substrates.[113,178,181] It has also been shown that when these two CBMs are linked together genetically, a boost in affinity is detected [182]. This is a common phenomenon seen with binding proteins [183].

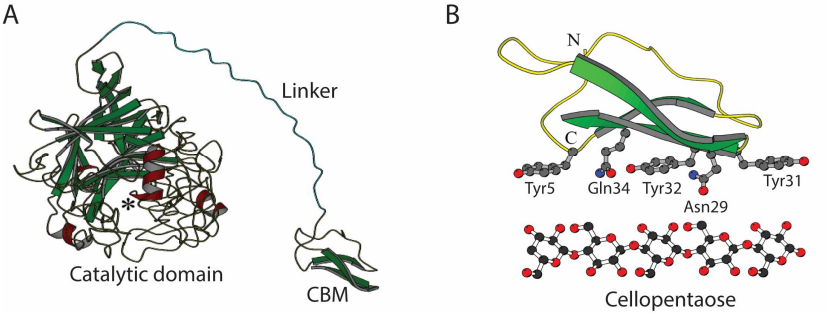


**Figure 5.** A) Structure of Cel7A cellulase showing the catalytic domain, the cellulose binding module and the linker between the two domains. (Image surtesy of Markus Linder.) B) The structure of CBM of Cel7A enzyme (CBM-Cel7A, adapted from the entry 1CBH in RCSB PDB), where the amino acids in the flat binding face are shown (Tyrosine (Tyr) 5, Tyr31, Tyr32, Glutamine (Gln) 34 and Asparagine (Asn) 29), and the structure of a five unit long cellulose chain, cellopentaose, aligned with the binding face of CBM-Cel7A.[184]

CBMs have been applied in biotechnology to bring function to cellulosic materials; cross-link cellulose with other materials such as starch, and to immobilize enzymes or proteins on paper[185–189]. In Publication (I) of this thesis we used a double cellulose binding module (DCBM) protein genetically fused to hydrophobin HFBI to stabilize surfaces and interfaces. In the fourth publication (IV) of this thesis we studied the role of the linker in the DCBM protein and its effect on the binding properties compared to the single CBMs. By altering the linker length it is possible to manipulate the protein binds onto NFC surface. This has been studied by Malho & Arola (manuscript under revision) and it has been shown that the linker length of the DCBM protein alone affects the film properties. Another example of the functionalization of NFC via HFBI-DCBM protein has been shown by Laaksonen et al. [190]. They used the ability of HFBI to exfoliate graphene [104] and combined it with DCBM ability to bind to NFC to create tough and strong nacre mimetic composites where the bifunctional protein connects the two materials at their interfaces.

In Publication IV we also show that the CBMs have preferences in binding to different substrates. We showed that the DCBM exhibit a boost in affinity when binding to BMCC compared to individual CBMs, which has been seen before[182]. On NFC

though, the CBM-Cel7A had higher affinity than any of the other proteins. We speculated that CBM-Cel7A has another binding site on NFC that does not exist on BMCC and which is not seen by CBM-Cel6A or the DCBMs. In this regard, NFC can also prove to be a very useful tool for cellulase research and opens up new insight and possibilities for the mechanisms on how these enzymes act on different substrates. HCl-CNC should also be potential for the study of cellulose degrading enzymes as well as CBMs alone. The pure crystalline cellulose nature of CNC would be useful for solely studying the enzyme activity on cellulose and CBM binding without the other materials that are present in NFC or lignocelluloses; namely hemicelluloses and lignin but also amorphous cellulose.[13,191–193]

# 2. Aims of the present study

The overall aim of this work was to study how nanocellulose can be modified and functionalized through biochemical routes.

1. How can specific affinity be used to introduce novel function to nanocellulose surface? (I)

2. Can nanocellulose be used to stabilize interfaces? (I)

3. Can nanocellulose be used to immobilize biomolecules and what are the benefits compared to existing methods? (II)

4. What is the role of hemicellulose in NFC matrixes? (III)

5. How does structure of DCBM affect their adsorption properties onto nanocellulose and what are the benefits in using nanocellulose in binding studies as a substrate? (IV)

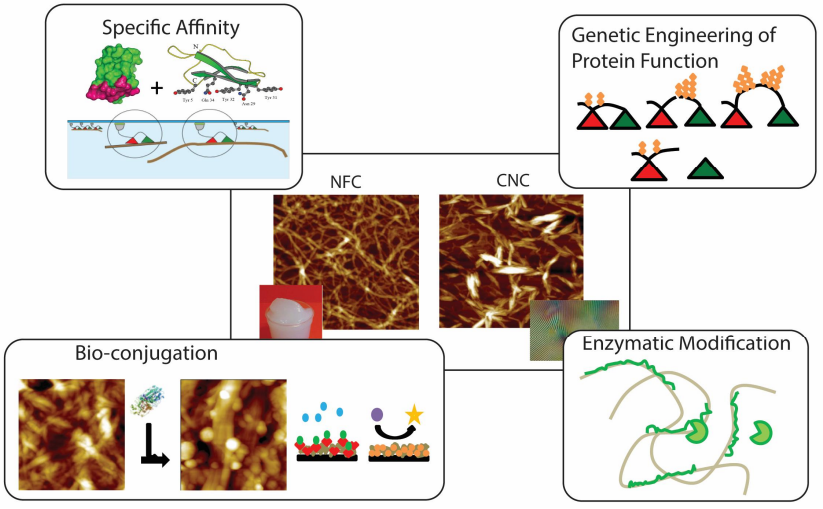


**Figure 6.** The different methods used in this study to accomplish biochemical modification and functionalization of nanocellulose.

# 3. Results and discussion

## 3.1 Using specific affinity of cellulose binding module (CBM) to introduce novel function to nanocellulose (Publication I)

In the first article we used a bi-functional recombinant protein, called the hydrophobin double cellulose binding module protein (HFBI-DCBM), to introduce amphiphilic function to nanocellulose surface. In HFBI-DCBM there are two CBM from the two major cellulases of the filamentous fungus *Trichoderma reesei*, Cel7A and Cel6A, and a class II hydrophobin, HFBI, of *T. reesei* (Figure 7).[194] The binding properties of the fusion protein to nanocellulose were studied using tritium labelled protein and the protein's ability to self-assemble at interfaces was studied in a Langmuir trough. The structure of the interfacial films that the HFBI-DCBM - nanocellulose complexes formed was studied by atomic force microscopy (AFM). The effect of nanocellulose on the strength of the interfacial film was studied by surface shear and dilatational rheology. The effect of nanocellulose on emulsion stability and structure was studied by different microscopy methods and by calculating the emulsion stability index.

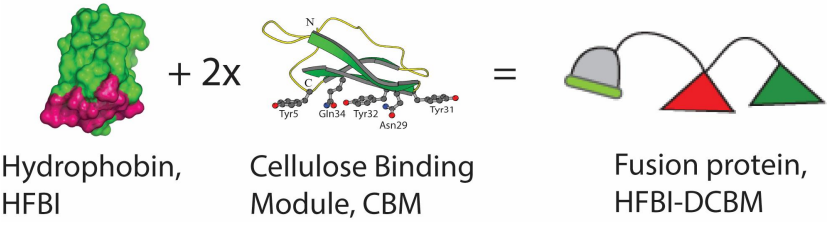


**Figure 7.** A schematic illustration of the components of the fusion protein HFBI-DCBM. HFBI structure[195] (PDB entry 2FZ6) is presented with hydrophobic to hydrophilic surface coverage of amino acids showing hydrophilic residues in green and hydrophobic residues in pink (the hydrophobic patch). The stick model of CBM is that of Cel7A cellulase[184] (PDB entry 1CBH). The two different CBM in the fusion protein are presented in different colors, CBM-Cel6A is in red and CBM-Cel7A is in green.

The binding capacity for HFBI-DCBM on NFC was very similar to what has been previously observed for an individual DCBM-construct on bacterial cellulose. It has also been previously shown that affinity can be increased by placing two CBM in tandem compared to using single CBM.[182] This was why DCBM was chosen as the fusion partner for the HFBI protein.

The rate of release of HFBI-DCBM was investigated to understand how stable the modification on nanocellulose would be under changing conditions. The results showed that in two hours, only a small amount of HFBI-DCBM came off from NFC

surface due to dilution indicating that the desorption rate is very slow. The exchange rates of the single CBM have been studied previously and it has been shown that the binding of Cel7A-CBM is truly reversible on BMCC substrate in contrast to Cel6A-CBM that showed minimal exchange on BMCC during the time course of the experiment. In this study for the HFBI-DCBM a non-complete or very slow adsorption could thus be expected. This was seen as a good property since in materials uncontrolled release of the DCBM moiety of the fusion protein could cause problems.

The ability of HFBI-DCBM modified NFC and CNC to assemble at the air-water surface was studied in a Langmuir trough. The formed surface films were visualized by atomic force microscopy (AFM) (Figure 8), which were lifted on graphite by Langmuir-Schaefer -method (LS). The protein modified NFC and CNC readily assemble at the surface. In contrast no assembly of NFC or CNC was seen if protein was not used. These results showed that the protein does bring function to nanocellulose surface and that it can be used in the formation of nanocellulose interfacial films.

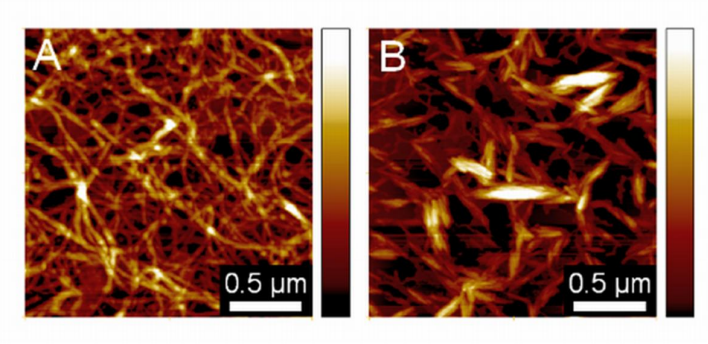


**Figure 8.** AFM images of Langmuir-Schaefer -films of A) a self-assembled film of NFC + HFBI-DCBM and B) a self-assembled film of CNC + HFBI-DCBM at the air-water surface. Scan size 2x2 µm, height 30 nm.

To study the effect of NFC in the surface self-assembled films, we performed surface shear rheology experiments at the air-water surface. The results of the HFBI-DCBM + NFC -films were compared to HFBI-DCBM and HFBI films. All the films showed a high elastic modulus, G', compared to the viscous modulus, G'', indicating that the films were very elastic. The highest value for G' was gained for HFBI film and it formed faster than the other films. The results showed also that NFC does not contribute to the strength of the film indicating that it is not incorporated in the film structure but rather under the protein film. This was also seen in the AFM images. NFC caused variation in the results suggesting an uneven distribution of the fibrils under the films.

Interfacial dilatational rheology experiments were used to study the NFC assembly with and without HFBI-DCBM at air-water surface and oil-water interface. Table

1 summarizes the dilatational rheology results. A low phase lag for all film types was observed implying a formation of an elastic film. Surface tension was significantly lowered by HFBI-DCBM on both air-water surface and oil-water interface, which indicates that the fusion protein adsorbs on both. The values gained for surface tension of HFBI-DCBM + NFC -films were comparable to those obtained for the protein only films indicating that the incorporation of NFC did not affect the proteins ability to self-assemble. The adsorption of NFC alone did not lower the surface tensions of either of the surfaces as much as the protein did which indicates that the protein has a stronger tendency to adsorb on the surfaces than NFC. NFC lowered the interfacial tension of the oil-water interface more than the surface tension of air-water surface, which indicates that NFC has a larger tendency to adsorb on oil-water interfaces. The dilatational moduli, E' and E'', for NFC at oil-water interface were also significantly larger than the ones for air-water surface indicating that NFC forms a stronger interfacial film on oil-water interface than at air-water surface. The values for dilatational elastic modulus (E') at air-water surface for pure protein were the highest indicating that HFBI-DCBM alone forms the strongest film with strongest intermolecular interactions. The lowest values were gained for pure NFC at air-water surface indicating insignificant self-assembly behavior but rather precipitation of fibrils unevenly. The mixed film of fusion protein and NFC gave intermediate values at air-water surface indicating some disturbance of the protein film structure by NFC. At the oil-water interface E'-values were generally much lower compared to the air-water surface, which indicates that the protein film structure or the interactions formed are different on the different interfaces/surfaces. The fusion protein alone gave the highest E'-values at oil-water interface. This behavior is comparable to the air-water surface. The behavior of fusion protein + NFC -films was different on oil-water interface compared to air-water surface giving the lowest E'-values at oil-water interface. This indicates that NFC disturbs the protein film structure more at oil-water interface than at air-water surface. The result was expected since NFC alone forms a stronger film at oil-water interface than at air-water surface.

**Table 1.** The surface and interfacial dilatational moduli of HFBI-DCBM, NFC, and their mixture -films at air-water surface and oil-water interface. |E| represents the complex modulus, E' is the elastic modulus and E'' the viscous modulus of the films.

| | Surface tension (mN $m^{-1}$) | \|E\| (mN $m^{-1}$) | E' (mN $m^{-1}$) | E'' (mN $m^{-1}$) | Phase lag (deg) |
|---|---|---|---|---|---|
| HFBI-DCBM (a-w) | 57 | 235 | 243 | 6 | -1 |
| HFBI-DCBM (o-w) | 9 | 33 | 32 | 5 | 8 |
| NFC (a-w) | 63 | 8 | 8 | 0 | 1 |
| NFC (o-w) | 15 | 21 | 20 | 4 | 11 |
| HFBI-DCBM + NFC (a-w) | 53 | 146 | 145 | 16 | 6 |
| HFBI-DCBM + NFC (o-w) | 9 | 14 | 14 | 1 | 5 |

As was shown above the self-assembly of HFBI-DCBM and NFC lead to interfacial films at oil-water interface. Thus we investigated how the formation of such films could affect emulsion properties. Emulsions of water and hexadecane were prepared in the presence of HFBI-DCBM and/or NFC and the resulting emulsion structures were studied by microscopy. The emulsions were confirmed to be oil-in-water type by staining the oil face. In emulsions made by only HFBI-DCBM or both NFC + HFBI-DCBM the droplet size was much smaller than in those formed by NFC alone. In the emulsions where NFC was present it could be seen covering the oil droplets and when the amount of NFC was increased it filled the continuous phase forming a network where the oil droplets were trapped. Similar structures were observed for CNC with and without HFBI-DCBM.

The stability of the emulsions was studied by following the separation of the emulsion phase from the aqueous phase. The results were presented as emulsion stability indexes (ESI). ESI is calculated from the volume of separated water and the total amount of water in the system (equation (1) in Materials and Methods, Section

4.3). Emulsions with NFC or CNC were prepared with and without HFBI-DCBM and a control emulsion with only HFBI-DCBM was also prepared. NFC containing emulsions were more stable than those made with CNC. In general the stability of NFC-containing emulsions was dependent on the cellulose concentration. A further enhancement of the stability was achieved by the addition of HFBI-DCBM to the system. Full stability (100%) of emulsion was achieved in conditions where NFC concentration was ~1.25 g $L^{-1}$ and HFBI-DCBM concentration was 1 g $L^{-1}$. With only NFC-containing emulsions at the studied concentration range (up to 2 g $L^{-1}$) the most stabile emulsion had a volume of 80%. HFBI-DCBM alone functioned quite well in stabilizing the emulsions with concentrations higher than 1 g $L^{-1}$ but the equilibrium volumes above 80% could not be achieved. The ESI value for control emulsion made with 1 g $L^{-1}$ HFBI and 1 g $L^{-1}$ NFC was 54%. The only variable that showed a clear dependency on the ESI values was the amount of bound protein that could be calculated from the binding isotherm. This indicates that the best emulsion stability occurs when maximal binding capacity of HFBI-DCBM to NFC is achieved.

In contrast to previous studies where NC materials have been used in emulsion stabilization, here we introduced a specific function of self-assembly to nanocellulose via specific function of DCBM fusion protein, and created stronger surface and interfacial films of nanocellulose than could be achieved without the specific protein functions.[107,109,110,112] The study shows that emulsions can be stabilized further by the functionalization of nanocellulose compared to nanocellulose alone or the modifying protein alone.

## 3.2 Using NFC as a platform for protein conjugation and the bioactive film formation of the conjugates (Publication II)

Protein immobilization onto surfaces is widely used in many biotechnical applications ranging from the ability to reuse enzymes in industrial processes to diagnostic methods largely relying on antigen/anti-body immobilization[196–200]. The most significant drawbacks of protein immobilization methods are that surface chemistries are often very slow relying on diffusion, immobilization effectiveness is restricted by steric hindrance of immobilized molecules, protein unfolding on surfaces, and poor control of the orientation of the immobilized molecule all causing loss of the bioactivity. Surface chemistries are also problematic to scale up for large surface areas.[159,196,198,201–204]

In the second publication of this thesis we took advantage of NFC intrinsic properties of dispersing to aqueous solutions and to form nanoporous films from solutions to immobilize enzymes and to form bio-active surfaces from the conjugates (Figure 9). We used three different bio-conjugation methods in bulk depending on the NFC-derivative, used spin-coating technique[123] to form bio-active surfaces of enzyme-NFC conjugates, and tested how the structure of NFC-derivative affected the formation and activity of multiple layers, analyzed the bioactivity on the films with different number of spin-coated layers, and investigated the structure of the films by AFM.

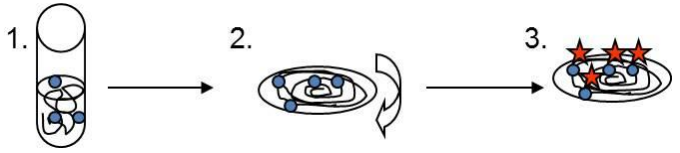


**Figure 9.** A schematic illustration of the bioconjugation process of a biomolecule to NFC. Step 1: Conjugation is done in solution. Step 2: Bioactive films are readily formed by spin-coating. Step 3: Bioactivity is detected.

The three different NFC-derivatives were; epoxy-modified (epoxy-NFC), amine-modified (amine-NFC), and carboxylic acid-modified (TEMPO-NFC). The amount of active groups on each derivative differed from one another. The degree of substitution (DS) for epoxy-NFC was 0.061, for amine-NFC 0.2 and for TEMPO-NFC 0.9. The DS-values gave an indication for us on how well the immobilization should work for each derivative. Alkaline phosphatase (AP) was chosen as an immobilization partner because it gives a colored reaction. The reaction yields and the success of the spin-coating of films were measured as the amount of bound AP per mass of NFC. Spin-coating was also used to create multiple layers of bioactive films to study the porosity of the NFC films and to add bioactivity onto the films.

The results showed that all the immobilization reactions were successful and that multiple layers of bioactive films could be formed by all conjugates adding activity onto the previous layer (Figure 10). AFM revealed that the structure of the bioactive films in general were very different from one another and were very much dependent on the starting material and the conjugation chemistries (Figure 11).

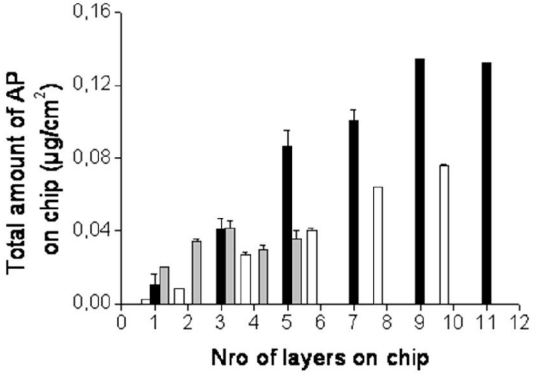


**Figure 10.** The amount of AP (µg) immobilized by each successive layer of the different NFC derivatives shown by the area of the chip ($cm^2$). White bars represent epoxy-NFC, grey bars represent amine-NFC, and black bars represent TEMPO-NFC.

The AFM images of all films formed by the non-conjugated NFC-derivatives and the AP-conjugated NFC-derivatives showed that the epoxy-NFC (Figure 11A) and the amine-NFC (Figure 11C) were more heterogeneous in fibril dimensions than TEMPO-NFC (Figure 11E). The epoxy-NFC formed the most coarse and unevenly distributed films. TEMPO-NFC formed the finest films and amine-NFC formed more tightly packed films than epoxy-NFC.

The structure of the AP functionalized epoxy-NFC film (Figure 11B) did not differ from the original non-functionalized film. The AP molecules were not seen in the images and were most likely immobilized as monomers or dimes (AP molecule is a dimer in its active form). In AP functionalized amine-NFC (Figure 11D) and TEMPO-NFC (Figure 11F) however, larger aggregates of height of ~70 nm were seen. This was most likely caused by immobilization of AP clusters rather than single AP dimers (5–6 nm). The clustering of AP molecules in the case of TEMPO- and amine-NFC is very likely due to the chemistries used in the immobilization. The clustering is not possible in the epoxy-NFC case.

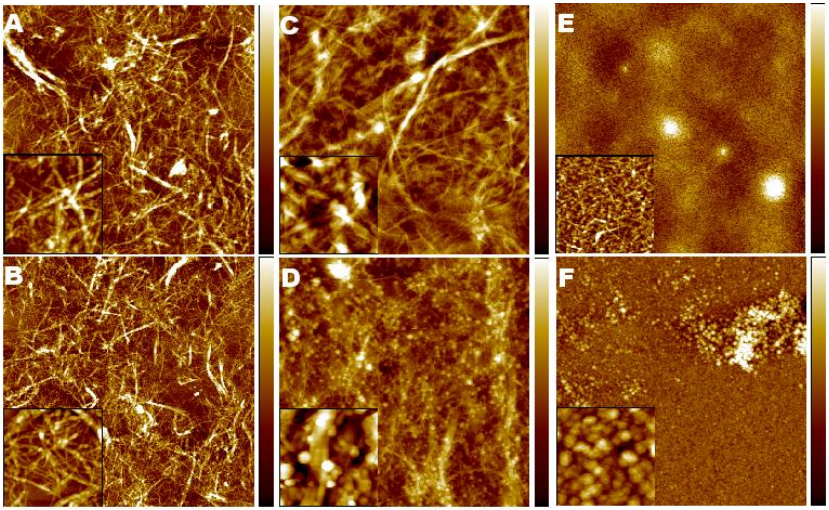


**Figure 11.** The AFM images of spin-coated layers of A) epoxy-NFC, B) AP functionalized epoxy-NFC, C) amine-NFC, D) AP functionalized amine-NFC, E) TEMPO-NFC, and F) AP functionalized TEMPO-NFC.

The film tightness, porosity and structure were also assessed by calculating the amount of AP (pmol) immobilized per amount of NFC (µg) in the films in each successive spin-coated layer (Figure 12). The data showed how the substrate for AP, a small molecule (*para*-nitrophenyl phosphate, *p*-NPP) is able to penetrate into the layers and react with AP. The data also showed that the most efficient of all the NFC derivatives was TEMPO-NFC; immobilizing the most AP compared to the amount of NFC spin-coated.

The amine-NFC formed the most tightly packed films, which caused the decrease in detection of AP per each spin-coated layer. From Figure 10 it was also evident that the amount of AP activity on the films could not be increased after four spin-coated layers for amine-NFC. In contrast, epoxy-NFC and TEMPO-NFC formed films that allowed the penetration of *p*-NPP and AP detection all the way through ten of more layers. Epoxy-NFC was shown to immobilize the least AP per NFC. Looking from the AFM image it seemed that the film structure was coarser than with the other derivatives allowing the penetration of *p*-NPP deeper into the multilayered film. With the TEMPO-NFC the swelling of the material is much better compared to non-modified or other chemically modified NFC. This is also a very good property for multilayering and protein immobilization allowing the formation of well hydrated loosely packed films with nanoporous structure. The hydrated environment is well suited for the proteins and the loose packing and porous structure allows diffusion of small molecules deep into the film structure.

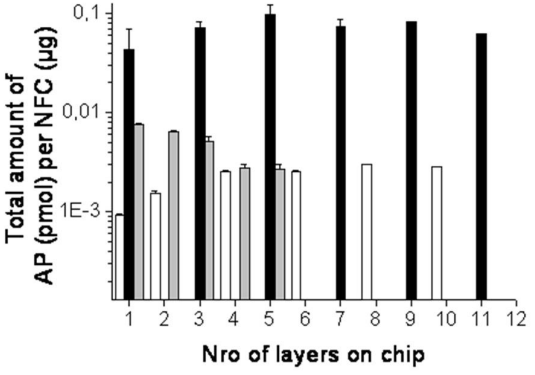


**Figure 12.** The amount of AP (pmol) immobilized by the different NFC derivatives (µg) per each spin-coated layer. White bars represent epoxy-NFC, grey bars represent amine-NFC, and black bars represent TEMPO-NFC.

There are two features which are thought to be very effective in protein immobilization and enzyme stability preservation; 1) multipoint covalent immobilization of proteins, and 2) carrier-bound cross-linked enzymatic aggregation [196]. In the case of epoxy-NFC the first criteria is met. In the case of amine- and TEMPO-NFC both criteria are met. This makes NFC a very good immobilization carrier for enzymes with the benefits of taking the immobilization chemistry from surface to bulk and being able to scale the process to large surfaces thanks to the ability of NFC to form films by simply casting.

## 3.3 Effect of xylan in NFC matrix

The formation of interacting nodes in percolating networks has been seen as the major explanation of the superior properties of NFC[22]. In the third publication (III) we address the question of how hemicellulose, a major component in NFC preparations from wood source, affects the NFC properties.

Depending on the origin and the processing history, NFC can contain almost 30% of hemicellulose (Publication III). In hardwood NFC, the main hemicellulose is glucuronoxylan[171]. As discussed earlier hemicelluloses aid the fibrillation process of pulp to MFC, which is most likely caused by the negative charges of hemicellulose that cause repulsion between fibrils[205,206]. To address the questions of how the hemicellulose is located in the fibril structure and how it affects NFC material properties in solution and in dry state, we used a specific enzyme, pI9 xylanase of *T. reesei*, to specifically remove xylan without affecting the cellulose structure[207]. We investigated the effect of xylan hydrolysis and mechanical mixing to the percolating gel network with rheology and to the film properties with extensional experiments. The morphology of fibrils was investigated by AFM and cryogenic transmission electron microscopy (cryo-TEM). To detect possible effects of xylanase treatment to cellulose structure we performed dynamic vapor sorption (DVS) studies.

The hydrolysis of NFC with only pI9 xylanase resulted in a release of 32-36% of the total xylan (~27 % of the material) as soluble saccharides from the material, corresponding to approximately 10% reduction of total mass. Physical agitation, i.e. mixing, did not affect the hydrolysis yield. These results indicated that xylan in hardwood NFC is only partly available for the enzyme.

AFM images and cryo-TEM images were obtained for all samples; NFC, xylanase treated NFC, mixed NFC, and xylanase treated mixed NFC. All in all the morphology and dimensions of the fibrils were not affected by the different treatments. Therefore from this data it is difficult to say how exactly xylan is associated with cellulose in the NFC matrix, whether it is as thin and evenly spread layer along the fibrils or only as loosely bound sparse patches.

DVS studies were done to verify that xylanase treatment did not result in the alteration of cellulose structure in NFC. It has been shown earlier that in relative humidity (RH) less than 75% the water vapor sorption capability of cellulosic materials is dictated by their crystallinity and above RH 75% by structural features such as their porosity[208]. All samples showed very similar water uptake profiles, thus xylanase treatment did not alter the crystallinity of NFC.

Small deformation stress sweep experiments were performed for all four samples. The elastic modulus (G'), viscous modulus (G''), phase angle (tanδ), and the critical stress at the onset of non-linear behavior were recorded. The systems were all elastic in nature. Xylanase treatment increased the elastic moduli and the critical stress value. However, mixing lowered the elastic moduli of both NFC samples in comparison to non-mixed NFC samples. The effect was more pronounced in the case of xylanase treated NFC. The effect of xylanase without mixing was interpreted as an increase in interactions between fibrils or as an increase in the number of

stronger cellulose-cellulose interactions (compared to xylan-cellulose), which resulted in a stronger percolating network. The mechanism of continuous mixing causing lowering of the elastic moduli was most likely related to less affective percolation throughout the gel due to the formation of flocs caused by the shear forces during mixing also seen previously[209]. Flocculation causes loss of global percolation in the gel and concentration of local interactions of fibrils. The xylanase treatment of mixed samples lead to an even larger decrease of elastic modulus, thus, the formation of flocs was apparently enhanced by xylanase treatment. These results indicated that xylan removal can be favorable to some extent but when shear forces are applied flocculation is greater and percolating network structure is lost more easily.

The elastic-to-viscous transition of the gels, i.e. the critical stress, studied by increasing the applied stress supported the findings from the above rheology experiments. The results followed the logic that mixing induces flocculation of fibrils causing loss of global interactions throughout the gel structure, which the xylanase treatment enhances, but if samples were at rest (no mixing), the xylanase removal made the gel network stronger.

NFC films of the differently treated samples were studied by extension experiments. Although the preparation protocol was identical different thicknesses were obtained for the different films and thus different densities. The reduction of thickness in the xylanase treated films was expected by the loss of mass due to hydrolysis. Also the mixing reduced the film thickness apparently by allowing a denser packing of the fibrils.

The measured parameters (Young's modulus i.e. stiffness, yield strength, ultimate tensile strength, and strain-to-failure) showed dependence to both mixing and xylanase treatment. In general, xylanase treatment decreased the values for mixed samples and increased the values for non-mixed samples, except in the case of strain to failure where xylanase treatment generally led to lower values. When NFC samples were not mixed during xylanase treatment, the films became stiffer and stronger. This was in line with the rheology data where gel strength was improved by the same treatment.

Opposite to rheology results, continuous mixing also resulted in stiffer and stronger films without xylanase treatment. Since the NFC samples not treated with xylanase were chemically identical, there must be other factors leading to improved properties. The thickness of the mixed NFC film compared to NFC without any treatments was lower. This indicates a better packing of fibrils in mixed samples, possibly caused by flocculation, and thus an increase in the interactions formed in the film. On the other hand, the xylanase treatment of mixed NFC samples further weakened the film properties indicating poorer interactions between the fibrils. The reduction in mass but not a markedly higher density implies also that the packing density of xylanase treated mixed NFC is poorer compared to NFC with only mixing and thus xylan seems to play a role in the packing of fibrils during drying. In all cases the strain-to-failure values were lower for xylanase treated NFC regardless of mixing. This showed that xylan has a role in fibril network extension in the plastic deformation region. Xylan mediated interactions between the fibrils apparently allow more creep and extension, while interactions between fibrils treated with xylanase

were stiffer but less flexible. Xylan, in this context, was beneficial and allowed better sliding of the fibrils against each other under tensile stress.

The results shown in Publication III support a major role of fibril network interactions in NFC properties, but also show that xylan as an additional component affects these interactions greatly and therefore the details of NFC performance in different applications. The results show also that xylan plays a key role in fibril stability in aqueous surroundings preventing for example fibril coalescence during mechanical mixing. In some cases the mechanical properties of materials can be enhanced by xylan removal, but considerations for the loss of stability of the fibrils and a better control of processing conditions may be needed.

## 3.4 Effect of linker length on DCBM binding properties and substrate dependency of CBM binding

As shown in Publication I, cellulose binding modules (CBM) can be used to functionalize NFC fibril surface. By genetic engineering and fusion protein technologies it is possible to introduce novel properties to NFC[190]. For application purposes it is important to know how the proteins function at the fibril surface.

It has been previously shown with CBM and DCBM (synthetic or produced in *E. coli*) on BMCC that the DCBM displays an increased affinity compared to CBM[182]. The role of the linker region in DCBM binding has not been studied previously. It has been speculated that the role of the linker between the catalytic domain and the CBM in the cellulase is to provide correct spacing of the two domains [210]. If the linker region is very rigid in structure and the CBM have specific binding sites on the substrate, the length of the linker can potentially play a very important role in the DCBM binding. In the fourth publication (IV) we addressed the question of how the linker region in the DCBM protein between the individual CBM affects the binding. We designed three different DCBM constructs where the linker length between the CBM was varied (Figure 13). DCBM-24 had the original linker of 24 amino acids that has been used by Linder et al. with one amino acid change of R to Q (13$^{th}$ amino acid of the linker)[182] to prevent the protein from trypsin cleavage. DCBM-12 had the first 12 amino acids of the original 24 amino acid linker, and DCBM-48 had a doubled 24 amino acid linker.

DCBM-12
RGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTS
TQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

DCBM-24
RGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTS
QPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

DCBM-48
RGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTS
QPATTTGSSPGPPGANPPGTTTTSQPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASG
TTCQVLNPYYSQCL

**Figure 13.** Amino acid sequences of the three different DCBM. Linker regions are in black, CBM-Cel6A sequence is shown in red, CBM-Cel7A sequence is shown in green.

After expression, purification and proteolytic cleavage of the original HFBI-DCBM, we verified the identity of the single CBM cleaved with papain from HFBI-DCBM-12 with amino acid composition analysis and matrix assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS). We also analyzed the extent of glycosylation of the linkers in DCBM cleaved by trypsin from HFBI-DCBM with MALDI-TOF. The results showed that the CBM-Cel7A and CBM-Cel6A were not glycosylated and that the digestion by papain leads to varying linker lengths attached on both sides of CBM-Cel6A, and CBM-Cel7A with no linker or only one or few amino acids of the linker. From MALDI-TOF it was also evident that DCBM-12 was not glycosylated but the other DCBM were heavily glycosylated; DCBM-24 by 14-25 and DCBM-48 by 34-50 glycan moieties. The glycosylation pattern is heterogeneous as expected for *T. reesei.*

For the binding studies, we labelled the five different proteins; DCBM-12, -24, -48, CBM-Cel7A, and CBM-Cel6A, with tritium ($^3$H) since this method is the most accurate and gives reliable results with a broad concentration range. We measured the binding isotherms of all proteins on BMCC and NFC. Figure 14 presents the start of the isotherms of all five proteins on BMCC and NFC. The Langmuir one site binding model was used to for fitting (Equation (2)) and relative partitioning coefficients ($K_r$) of the initial slopes for the binding isotherms were obtained (Equation (3)).

From the isotherms it was evident that the linker length affected the DCBM binding in the same way on both substrates; DCBM-24 having the highest affinity of the three, DCBM-12 having an affinity slightly lower than the DCBM-24 and DCBM-48 having the lowest affinity compared to the two other DCBM. These results clearly show that the linker length plays a role in the binding of the molecule. The most optimal linker length was the intermediate length with some glycosylation. The linker in DCBM-48 contained substantial amounts of glycan moieties and thus is most

likely to be more rigid in structure than the other two allowing less freedom for binding. Also the length is much longer than in the case of the others. The poorer binding of the DCBM-48 could be caused by geometrical issues or less favorable energetics connected with the binding event due to the rigid linker preventing optimal binding.

What was also evident from the binding isotherms was that on BMCC the doubling of the binding moiety causes an increase in affinity and the individual CBM both bind with lower affinity than the DCBM-24 and DCBM-12. This was in line with previous studies[182] and is a well-known phenomenon occurring with other binding events in nature[183]. A surprise in the results was seen with binding of the CBM with NFC. From the isotherms it can be seen that CBM-Cel7A binds with higher affinity to NFC compared to all the other proteins and that the doubling of the binding units does not increase the affinity but rather seems to hinder the binding of the one moiety (CBM-Cel7A) and/or increase the binding of the other moiety (CBM-Cel6A). This peculiar behavior could be explained by CBM-Cel7A having two distinct binding sites or two modes of binding on NFC, which are geometrically or energetically different from one another. By linking the CBM-Cel7A to CBM-Cel6A the other binding site or mode of CBM-Cel7A seems not to be available in the double form and thus the affinity is lower for the DCBM compared to CBM-Cel7A alone.

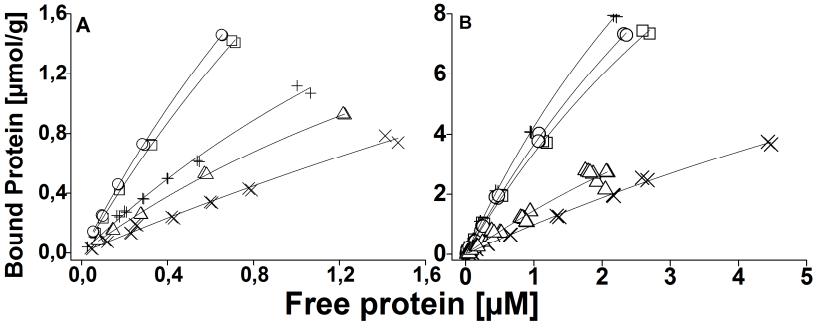


**Figure 14.** The initial slopes of the binding isotherms for the five different proteins on A) BMCC and B) NFC. (+) CBM-Cel7A, (x) CBM-Cel6A, (□) DCBM-12, (○) DCBM-24, (Δ) DCBM-48.

To be able to compare the binding energetics of the different proteins we can calculate Gibb's free energy of binding (ΔG, Equation (5)). For ΔG calculations reliable values for maximum binding capacity ($B_{max}$) are needed. To measure these, the binding studies were performed with higher concentrations of protein. The so called full binding isotherms for all the five proteins on both substrates are presented in Figure 15 as semi-logarithmic plots to illustrate how far or how close to the actual $B_{max}$-values the gained data were. From the full set of binding data it can be seen that CBM-Cel7A had the highest $B_{max}$-value of all the proteins on both substrates and clearly had a higher packing density on both substrates. It was also evident that

the maximum binding capacity for all other proteins except for DCBM-48 on both substrates was not reached. For DCBM-12 and -24 the $B_{max}$-values were very close to the actual $B_{max}$-values and could be used to calculate reliable ΔG-values. For the CBM the $B_{max}$-values are not accurate, thus the ΔG-values gained are merely directional.

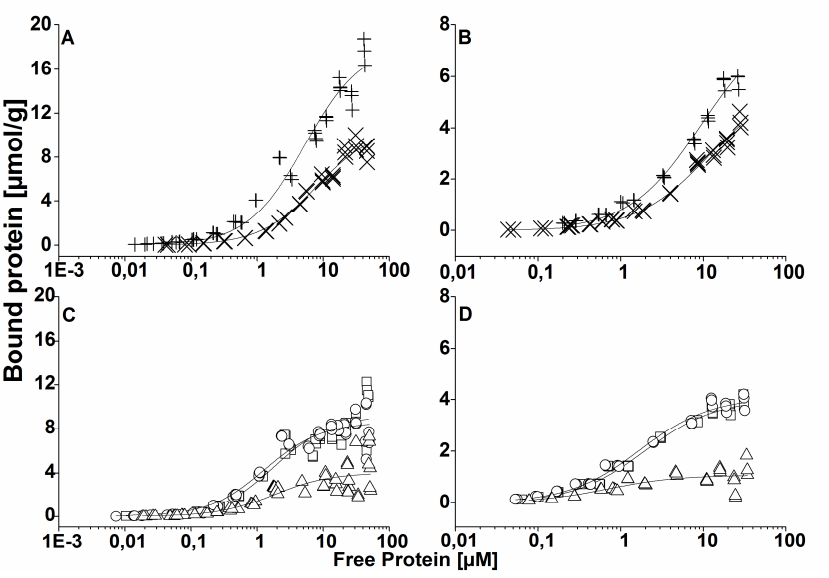


**Figure 15.** Semi-logarithmic plots of the CBMs on A) NFC and B) BMCC, and DCBMs on C) NFC and D) BMCC. (+) CBM-Cel7A, (x) CBM-Cel6A, (□) DCBM-12, (○) DCBM-24, (Δ) DCBM-48.

The $B_{max}$-values gained from the experimental data were accurate for the DCBM-48. For other proteins a theoretical minimum and maximum energies were calculated by using the accurate $K_r$-value gained from the initial slope of the beginning of the binding isotherm and using the $B_{max}$ gained from the data in Figure 15 and by over estimating the $B_{max}$-value to see the effect on energy when the $B_{max}$ is larger than measured data. The estimations were done by doubling and tripling the $B_{max}$-values gained from the full measured data. We refer to them as low, intermediate and high $B_{max}$-values. These values represent a range where the actual ΔG-values might lie and provide an estimate that can be used to compare the proteins to one another. This shows the importance of gaining accurate $B_{max}$-values for the calculations of the ΔG-values. The values gained from the initial slope for $k_d$ and $B_{max}$ are not accurate and thus not very good for estimating the ΔG-values for any binding event. On the other hand $K_r$-values gained from the same data are accurate due to their relative nature.

From Table 2 we can see that the energies of binding for the DCBM on both substrates are more favorable than those for the individual CBM. This shows that the coupling of the CBM together is energetically sensible. The energies of binding in the case of the CBM showed only slight differences in comparison between substrates or proteins. This suggests that the biding sites for CBM-Cel7A and CBM-Cel6A on both substrates are energetically equal and the difference for the binding affinity would come rather from geometry of the binding than from energetics of the binding. The binding energies of the different DCBM are also very close to each other and in their case also the difference in the binding properties must lie in the geometry rather than energy of the binding.

**Table 2.** Gibb's free energies (ΔG) for CBM and DCBM on BMCC and NFC with low, intermediate and high $B_{max}$-values.

| | $B_{max}$ | CBM-Cel7A | CBM-Cel6A | DCBM-12 | DCBM-24 | DCBM-48 |
|---|---|---|---|---|---|---|
| NFC | low | -30,7 | -28,2 | -31,8 | -32,1 | -31,7 |
| | inter | -29,0 | -26,5 | -30,1 | -30,4 | |
| | high | -28,0 | -25,5 | -29,1 | -29,4 | |
| BMCC | low | -29,6 | -28,4 | -32,7 | -33,0 | -33,9 |
| | inter | -27,9 | -26,7 | -31,0 | -31,3 | |
| | high | -26,9 | -25,7 | -30,0 | -30,3 | |

Since the peculiar binding properties of CBM-Cel7A could not be explained by the energetic differences of the binding sites, differences in substrate structure was interrogated: i) NFC has a nanosized fibril structure that could geometrically promote the affinity of CBM-Cel7A but not CBM-Cel6A, ii) NFC contains ~27 % xylan which could serve as another substrate for CBM-Cel7A but not CBM-Cel6A, iii) the crystal structure and the ration of crystalline to amorphous cellulose is different in NFC compared to BMCC and this could cause a difference in the binding of CBM. All or some of these could potentially affect the binding site geometry for the two proteins.

To test if the nanoscale structure of the NFC promoted the CBM-Cel7A binding we studied the protein binding on the pulp from which the NFC originates from. There were no differences compared to NFC binding noted. Thus, we concluded that the binding of CBM-Cel7A is not promoted by a nanoscale effect caused by NFC structure or processing.

Next, to test the effect of hemicellulose, we performed a binding experiment of CBM-Cel7A on NFC that was treated with pI9 xylanase, which has some 30 % less xylan compared to non-treated NFC. The hydrolysis was expected to reveal cellulose surface and promote CBM binding. To what extent, it was hard to assess. On the other hand, if CBM-Cel7A would bind to xylan, the effect of xylan removal on the binding would be smaller than if it bound only on cellulose surface. We saw that by enzymatically removing xylan from NFC we increased the binding of CBM-Cel7A by 20 %. This is a substantial increase and thus it does not sound plausible that

xylan in NFC would cause the higher affinity of the CBM-Cel7A compared to DCBM. If CBM-Cel7A would have an affinity towards xylan, the hydrolysis should lower the amount of bound CBM on xylan and at the same time increase the binding to cellulose. The difference in binding in this case would not be expected to be very large expecting that the surface are of fibrils are not changed dramatically by xylan hydrolysis. In Publication (III) we did not see any morphological or structural changes of fibrils associated with the xylan hydrolysis.

To assess whether the crystal structure affects the binding is harder. What we could do was to study the exchange rates of the different proteins on the different substrates. This was done by recording the time it took for the $^{3}$H-labelled protein to exchange from the surface of the substrate to a non-labelled one at equilibrium. We performed the experiment to all five proteins on BMCC and NFC. The results are presented in Figure 16 and they show that both CBM exchange with a relatively fast time (<600 min) to 50 % from the original amount of bound $^{3}$H-protein from BMCC. For CBM-Cel7A this has been shown previously but for CBM-Cel6A different results have been shown in different studies [113,181]. In our experiments the results suggest that both CBM exchange fully and the bindings were clearly reversible on BMCC. A different result was obtained with NFC; the individual CBM did not reach the 50 % exchange during the time course of the experiment and they showed only partial exchange suggesting that the exchange rate of the binding is much slower on NFC that BMCC.

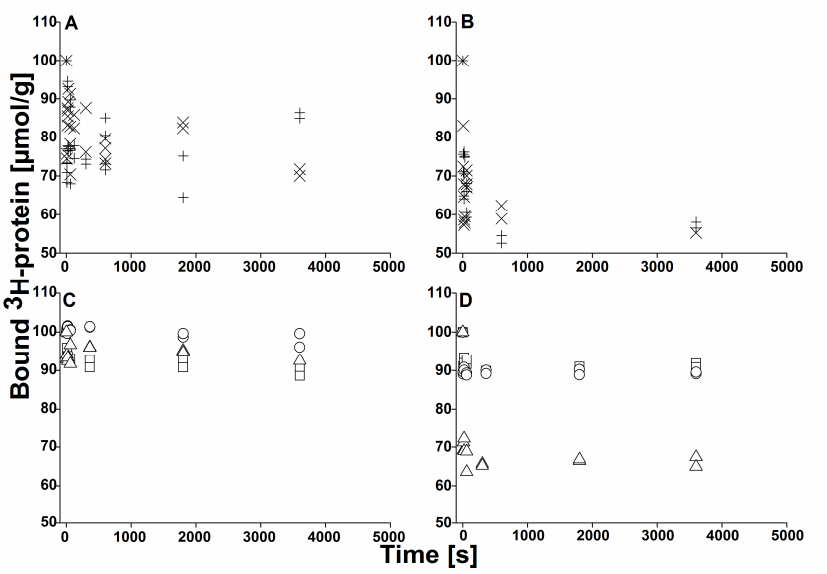


**Figure 16.** The exchange of $^{3}$H-labelled protein to non-labelled protein. CBM on A) NFC and B) BMCC, and DCBM on C) NFC and D) BMCC. (+) CBM-Cel7A, (x) CBM-Cel6A, (□) DCBM-12, (○) DCBM-24, (Δ) DCBM-48.

For DCBM the exchange to 50% was not seen on either substrate suggesting that the exchange rate is greatly affected by linking of the CBM together. For BMCC; for DCBM-24 and DCBM-12 the first 10 % of the exchange was very fast and there after the exchange was very slow with no detectable changes during the experiment. For DCBM-48 the same fast exchange in the beginning until 30% was seen, but there after no detectable changes were seen. On NFC; the exchange of DCBM-24 was so slow that no changes were detected during the time course of the experiment. For DCBM-12 and DCBM-48 the exchange was fast for the first ~10% and then so slow that no changes occurred during the experimental time.

From the exchange experiments it can be concluded that the proteins experience NFC and BMCC as different substrates whether they are linked together or not. It also seems that linking dramatically affects the rate of exchange of the proteins on the surface making it slower. For the cellulases with CBM this has been previously shown and has been attributed as the cause of the CBM [211,212]. DCBM exchange with a different extent from NFC than BMCC but not fully on either. It seems that desorption of the DCBM from BMCC and CBM from NFC have one very fast component and another extremely slow component. The DCBM-24 desorption is all in all extremely slow. The DCBM-12 and DCBM-48 on the other hand still seem to have a fast component that can be measured in combination with a slow component. The linker length has a clear effect on desorption but it does not follow the same logic on the two substrates. On BMCC the extent of exchange is largest for the longest linker, DCBM-48. For the shortest, DCBM-12, and the intermediate, DCBM-24, extent of exchange is lower. For NFC the DCBM-24 exchange is not seen at all, and for the other two, DCBM-12 and DCBM-48, the exchange levels are similar.

# 4. Materials and methods

## 4.1 Nanocellulose

### 4.1.1 Nanofibrillated cellulose (NFC)

Never-dried bleached birch kraft pulp was fluidized with 1600 bar pressure 10 times to yield NFC. It was prepared by and gained from the Finnish Centre for Nanocellulosic Technologies. Total enzymatic hydrolysis of the material (Publication III) yielded a cellulose content of ~71 % and the hemicellulose content was ~27.3 %. The insoluble fraction of the material was ~1.7 %. The AFM images of the material (I, II, III) showed that the width of the fibrils is heterogeneious and varies between ~5 to 40 nm. The length of the fibrils in in micron scale.

### 4.1.2 Cellulose nanocrystals (CNC)

CNC used in Publication (I) of this thesis were prepared as described previously [13], using Whatman 541 ash-less filter paper (Whatman, UK) as the starting material. They were received from Dr. Eero Kontturi. The surface of HCl hydrolyzed CNC is not modified by the acid hydrolysis and they have the natural surface composition of cellulose crystallites. In the first publication (I) it was essential that the cellulose surfaces were not modified because that could affect the specific binding of the DCBM protein. The diameter of the single crystals was approximately 5 nm and length was ~100 to 300 nm as seen in AFM.

### 4.1.3 Bacterial microcrystalline cellulose

Bacterial microcrystalline cellulose (BMCC) or bacterial cellulose (BC) was obtained from Nata de Coco, by first grinding, then washing with water and sodium acetate buffer pH6, and then homogenizing with a mechanical stirrer. The end product had a solid content of which had a solid content of 2.55 $gL^{-1}$, and was used for the binding studies along with NFC. The diameter of the BC were shown to be 5-7 nm and bundled to larger ~20nm fibrils.[91] The length of BC is in micron scale. The crystallinity of BC is generally higher than that of wood derived cellulose materials. The BC used in our studies was shown to be mostly crystalline.[91]

## 4.2 Used proteins

### 4.2.1 HFBI-DCBM, DCBM, and CBM

The fusion protein HFBI-DCBM used in the first publication (I) of this thesis consisted of an N-terminal hydrophobin linked to two CBM in series (sequence in Figure 14)[194]. The class II hydrophobin HFBI was from *Trichoderma reesei* and the two CBM were those of Cel7A (first in the sequence) and Cel6A (second) cellulase also found in *T. reesei*. The different modules were connected by polypeptide linkers as reported earlier. The fusion protein was produced by recombinant means in *T. reesei* and purified by aqueous two phase extraction as described previously[194]. The protein was further purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) using a water acetonitrile gradient with 0.1% trifluoro acetic acid (TFA).

The three different DCBM were all produced as HFBI-DCBM fusions in *T. reesei* to aid the purification. The proteins were purified as described by two phase extraction and RP-HPLC. The amino acid sequences of all HFBI-DCBM proteins are shown in Figure 17. Three modifications were made to the original HFBI-DCBM sequence. First, a tobacco etch virus (TEV) protease site (ENLYFQG) was introduced in the beginning of the linker between HFBI and CBM-Cel6A for a specific cleavage of the HFBI[213]. Second, the methionine residue (M) in the linker between HFBI and CBM-Cel6A was replaced by an arginine (R). This was done to introduce a trypsin cleavage site into the linker just in case if the TEV site would not function. Third, the arginine (R) in the linker between the two CBM was replaced by a glutamine (Q) to avoid trypsin cleavage.

Trypsin (sequencing grade modified trypsin, Promega) was used to gain the DCBM from the HFBI-DCBM (Figure 13) and papain (Promega) was used to gain CBM from HFBI-DCBM-12. The reactions were followed by reversed phase ultra-high performance liquid chromatography (RP-UPLC) using a 2.1 x 100 mm, 1.7 µm, C4 Acquity BEH300 prST column and an Acquity I-Class system with a photodiode array detector (Waters, MA, USA) and a linear water acetonitrile gradient with 0.1% TFA. The preparative RP-HPLC was used to purify the DCBM and the CBM from the reaction mixtures.

HFBI-DCBM
SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA
PGASTSTGMGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTSRPATTTGSSPG
PTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

HFBI-DCBM-12
SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA
ENLYFQGPGASTSTGRGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTSTQSH
YGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

HFBI-DCBM-24
SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA
ENLYFQGPGASTSTGRGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTSQPAT
TTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

HFBI-DCBM-48
SNGNGNVCPPGLFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALLCQTAVGA
ENLYFQGPGASTSTGRGPGGQACSSVWGQCGGQNWSGPTCCASGSTCVYSNDYYSQCLPGANPPGTTTTSQPAT
TTGSSPGPPGANPPGTTTTSQPATTTGSSPGPTQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL

**Figure 17.** The amino acid sequences of all HFBI-DCBM fusion protein. HFBI sequence is shown in blue, linker regions are shown in black, CBM-Cel6A is shown in red, and CBM-Cel7A is shown in green. The modified amino acids are shown in pink and added TEV site is shown in orange. The first sequence is that of HFBI-DCBM used in Publication I. The second, third and fourth are those of HFBI-DCBM-12, -24, and -48 respectively.

### 4.2.2 Alkaline phosphatase

Alkaline phosphatase (AP, E.C. 3.1.3.1.) is an esterase that catalyzes the hydrolysis of a bond between phosphor and oxygen in for example monoesters. It is routinely used as a fusion partner for example with antibodies or antigens in enzyme-linked immunosorbent assays (ELISA). When it hydrolyses a small molecule, *para*-nitrophenyl phosphate (*p*-NPP), the product of the reaction, *para*-nitrophenyl, adsorbs light strongly at A405 and can be quantified by a spectrophotometer. The amount of AP in a reaction can be gained by comparison to a standard curve of known amounts of AP.[214]

### 4.2.3 pI9 xylanase of *Trichoderma reesei*

The pI9 xylanase of *T. reesei* is one of the two major xylanases produced by the fungus. This enzyme has a specific activity towards xylan and does not hydrolyze cellulose or other polysaccharides. The pI9 xylanase has been shown to hydrolyze xylan to xylose and 2–7 unit xylo-oligomers.[207]

#### 4.2.3.1 Xylans

Xylans are hemicelluloses composed of a β-1-4-linked unhydroxylopyranose unit backbone. Xylans are heteropolysaccharides that in addition to xylose can contain arabinose, methylglucuronic acid and acetic acid substituents. They are abundant in especially hardwood and annual plants.[137,171]

## 4.3 Emulsion stability index

The emulsion stability indexes were calculated as is presented in Equation (1).

$$ESI = \left[1 - \frac{V_{H_2O,separated}}{V_{H_2O,total}}\right] \times 100\% \quad (1),$$

where $V_{H_2O,\ separated}$ is the volume of separated water from the emulsion phase and $V_{H_2O,\ total}$ is the total volume of the sample.

## 4.4 Langmuir trough

A Langmuir trough is an instrument used to create and study monolayers of materials at surfaces and interfaces. A surface is an interface between material and vacuum, and interfaces exist between two different liquids, two different solids or between a liquid and a solid, assuming they are not soluble. These surfaces and interfaces have a surface or an interfacial energy associated with them. The surface and interfacial energies can be measured as surface and interfacial tension or pressure.

Monolayers formed by, for example detergents, particles or proteins are of great importance in many industrial applications such as emulsion and foam technologies as discussed earlier. The details of the molecular interactions and the behavior of the molecules at the surface or interface can be studied in the Langmuir trough equipped with a pressure sensor (Figure 18) and a probe to measure surface tension, e.g. Wilhelmy plate or Du Noyü ring. A Langmuir film, i.e. a monolayer of molecules, can be formed by adsorption of material form the bulk. The change in surface pressure that is caused by the adsorption is measured by the pressure sensor. The monolayer that is formed on the surface or interface can be compressed by two barriers. From the pressure-area isotherm the average area of one molecule in the monolayer can be calculated if the amount of molecules at the surface and the total area they cover is known. It is also possible to gain the state (i.e. gas-, liquid-, solid-like) of the molecules in the monolayer from the isotherm.[215]

For many purposes such as microscopy imaging it is useful to be able to lift the monolayer onto a solid support from the trough. Depending on which side of the monolayer, hydrophilic or hydrophobic, is displayed on top of the support, two types of films can be created; Langmuir-Blodgett film (LB-film, Section 4.4.1) or Langmuir-Schaeffer film (LS-film, Section 4.4.2).

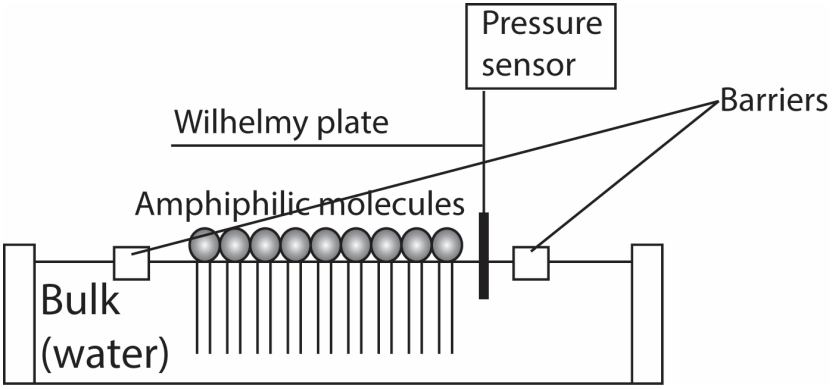


**Figure 18.** A schematic illustration of a Langmuir trough with compression barriers, a pressure sensor and a Wilhelmy plate.

### 4.4.1 Langmuir-Blodgett films

If the film is lifted from the bulk hydrophilic side phasing the support (Figure 19) the film is referred to as Langmuir-Blodgett film (LB-film). The film can be formed on the same support multiple times and in this way bilayers can be formed.[129,216]

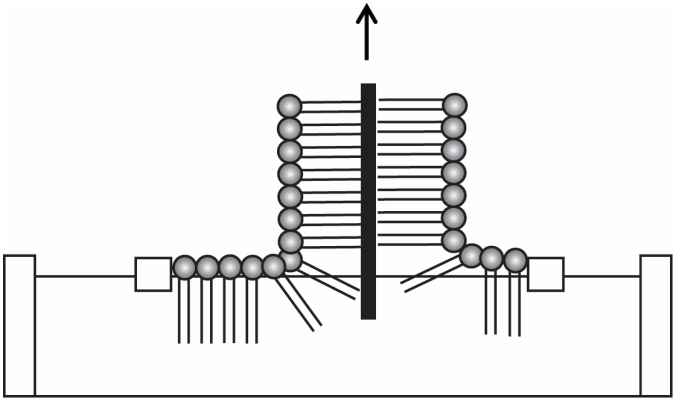

**Figure 19.** A schematic illustration of the preparation of LB-films.

### 4.4.2 Langmuir-Schaffer films

It the film is lifted with the hydrophobic side facing the solid support (Figure 20) the film is referred to as Langmuir-Schaeffer film (LS-film). This technique was used in the first publication of this thesis (I).[217]

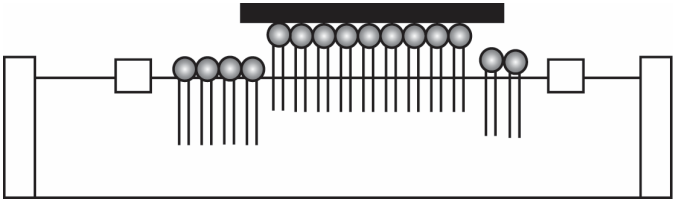

**Figure 20.** A schematic illustration of the preparation of LS-films.

## 4.5 Atomic force microscopy

Atomic force microscopy (AFM) is a powerful tool proven to be very useful in imaging, especially with regard to biological samples.[102,218–220] The biggest advantage of AFM in comparison to electron microscopy methods is that imaging can be done in ambient conditions and either in liquid media or in air. This makes it possible to image biomolecules in their natural environment. Another advantage is that no staining of the sample is needed but the sample needs to be attached on a solid support.

The principle of AFM is shown in Figure 21.[221] A laser is reflected on a cantilever's free end, which is carrying a tip. When the tip comes into contact with a sample surface the cantilever is bent due to the forces acting between the sample and the tip. The bending causes deflection in the laser beam and this is recorded by a photosensitive detector (photodiode). The sample is scanned by the tip line by line in XY-direction and a feedback loop between the photodiode and the Z-scanner provides the height information during scanning. The height of the sample is adjusted by the Z-scanner to keep the laser deflection constant. The Z-piezo movement is in the sub-nanometer range and sub-Ångstöm movement of the cantilever can be detected by the optics of the system.

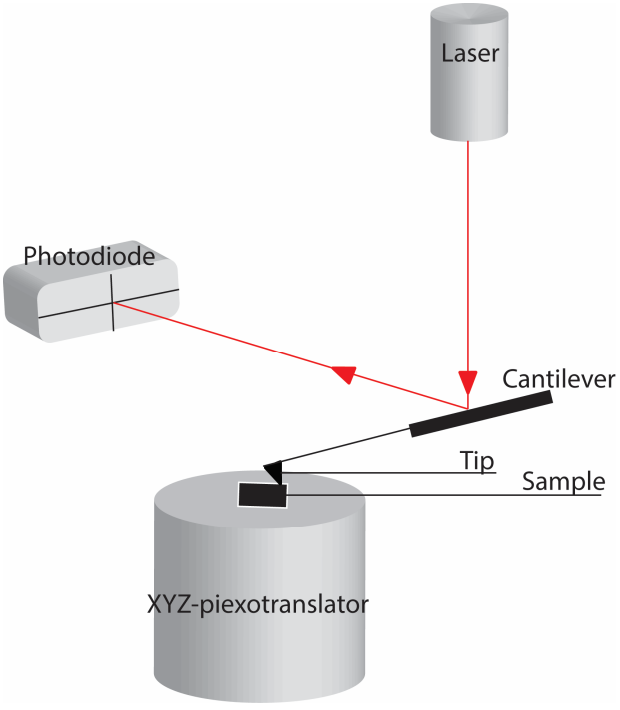


**Figure 21.** The basic principle of AFM imaging.

The tapping mode AFM is more useful for imaging biological sample due to less damaged caused by the more gentle contact of the tip to sample compared to above described contact mode. In this method the cantilever is oscillating near its resonant frequency very close to the surface of the sample and the tip is briefly touching the surface during each oscillation cycle. During scanning, damping of the free amplitude of the cantilever ($A_f$) is controlled by altering of the set point amplitude ($A_{sp}$). The $A_f$ is a predetermined amplitude that is unique for each cantilever. The $A_{sp}$ is the amplitude of the cantilever when it is in contact with the sample. The ratio of $A_{sp}$ and $A_f$ describes the force that is applied to the sample and it is called the damping ratio. The damping in the $A_{sp}$ is kept constant during scanning and it is monitored by the feedback loop. The photodiode records changes in the received signal and they are corrected back to the predetermined set point value by the movement of the sample in Z-direction.

Tapping mode AFM (Nanoscope IIIa Multi Mode AFM, with E- and J-scanner, Digital Instruments/Veeco with NSCI5/AlBS cantilever, µMASCH, USA) was used in this thesis to image Langmuir-Schaeffer films of different proteins alone or in combination with nanocellulose (I), to image spin-coated films of NFC-derivatives and

films formed by NFC-derivatives conjugated with AP (II), and films prepared from NFC before and after xylanase treatment with or without mechanical shearing (III).

## 4.6 Rheology

Rheology is a study of flow and deformation of matter. Rheology describes the flow behavior of liquids and the deformation behavior of solids. Most materials lie in between two extreme ideal materials in their rheological behavior; the flow of ideal viscous fluids and the deformation of ideal elastic solids, thus making them viscoelastic liquids or viscoelastic solids comprising of both viscous and elastic portion. Viscoelastic solids behave differently in comparison to viscoelastic liquids depending on their rheology. Viscoelastic materials have a delayed response to the applied force and also when the force is removed.[51,222]

Small deformation time dependent oscillatory tests in shear rheology are used to examine interaction between molecules in all kinds of viscoelastic materials ranging from low-viscosity liquids to fairly rigid solids. These measurements are performed by deforming the sample by application of periodic oscillatory (sinusoidal) stress (σ) or strain (γ) at constant frequency (ω) and measuring the stress and strain respectively. When a sinusoidal shear force is applied the amplitude of the response and the delay in the phase angel (δ) will depend on the viscoelastic properties of the measured sample. In ideal elastic materials the stress and strain sinusoidal curves are in phase and the phase angle is 0°. In ideal viscous materials the stress and strain curves are out of phase and the phase shift angle is 90°. For viscoelastic materials the phase shift angle thus lies between 0° and 90°.

There are some parameters that are important for rheology; the complex shear modulus, G*, which results from the sinusoidal shear process and consists of both the elastic and viscous response of the material. The shear storage modulus, G', that tells how solid-like the material is and how much the material stores energy during application of force, and the shear loss modulus, G'', which describes how liquid-like the material is and how much energy is lost during the application of force. The loss modulus or the tanδ (tangent delta) is calculated as G''/G'. This describes the ratio of viscous and elastic portion in the material. If tanδ = 0, the material is fully elastic since G' >> G'', on the other hand if tanδ = ∞, the material is fully viscous since G'<< G''. If the two components, viscous and elastic, are exactly equal tanδ = 1.

In contrast to shear rheology, which is commonly used to study viscoelastic materials, the extensional rheology parameter that is gained from extension of flexure tests are referred to as E. In steady state measurements that are performed to solid materials to determine their strength yields the parameter E, i.e. tensile modulus, which is usually referred to as Young's modulus. The Young's modulus is determined in the linear-elastic region of tensile tests where the tensile stress and tensile strain or elongation shows a constant slope. The complex dilatational modulus, storage modulus and loss modulus are referred to as E* or |E|, E', and E'' respectively

and describe the material during the application of periodical extensional or flexure force, i.e. small deformation oscillatory tests also referred to as dilatational rheology, to distinguish the difference between the type of applied forces.

In Publication III small deformation oscillatory shear tests using cylinder and cup geometry (CC 15, gap between bob and wall 0.5 mm), with the stress controlled rheometer (StressTech, Rheological Instruments Ab, Sweden) were performed to investigate the effect of xylan in the NFC gel matrix. Xylan hydrolysis was performed by pI9 xylanase enzyme and the effect to the gel strength was compared to non-hydrolyzed samples. The effect of xylan in NFC matrixes in dry state was investigated by steady state tensile measurements using a mini tensile tester (Deben, UK), on solid self-standing films prepared from the same samples.

### 4.6.1 Surface and interfacial rheology

Rheology in three dimensional systems is called bulk rheology but rheology can also be performed in two dimensions, i.e. at surfaces or at interfaces. The tests done apply small deformation to the sample and the parameters that are gained are the same as in bulk systems applying small deformation. The G-values refer to interfacial or surface shear moduli and E-values refer to dilatational interfacial or surface moduli. Deformation applied at the surface or interface layers provide indirect information on the molecular interaction between and within the adsorbed layer of molecules such as protein. When sinusoidal oscillation with pre-defined strain amplitude is applied the surface film is sheared analogously to the bulk system and the gained parameters are accordingly similar.[223,224] The shearing is done by placing a light weight Du Noüy ring geometry carefully onto the surface.

In dilatational rheology experiments the volume of the bubble/drop is changed and the response of the surface tension is determined from the shape of a bubble/drop by Gauss-Laplace equation that is transformed into a set of first-order differential equations, which are expressed by the geometrical parameters of the bubble/drop profile. As a result sinusoidally oscillating the bubble/drop and recording the changes in surface area with a high speed camera we are able to obtain the surface/interfacial tension as a function of time in parallel to the change in surface area. A Fourier analysis allows the extraction of pairs of parameters, such as |E| and phase angle, and E' and E".[223]

In Publication I of this thesis the surface/interfacial shear and dilatational rheology methods were used to investigate the strength and nature of the interfacial and surface films formed by HFBI-DCBM in combination with NFC and CNC and compared to HFBI-DCBM, NFC and CNC alone. The investigated interface was hexadecane-water and the surface was air-water.

Dilatational surface and interface rheology of the protein films with and without NFC and CNC as well as the NFC and CNC films were studied using an oscillating bubble or pendant drop, respectively. The air bubble in water and the water droplet in oil were formed and the surface/interfacial tension was followed until equilibrium

was reached using a contact angle measurement device (CAM 200, KSV Instruments Ltd., Finland). The equilibrated air bubble or water droplet was oscillated in a sinusoidal manner using a pulsating drop module (KSV PD 100, KSV Instruments Ltd., Finland). The change in surface area and tension of the bubble/droplet were determined from a set of oscillations (0.1 Hz) and then the measurement was repeated a few times. The rheological parameters were determined from the data with the instruments own software (OscDrop2008, KSV Instruments Ltd., Finland).

Small deformation shear oscillatory measurements were performed at air-water surface to protein films with and without NFC and CNC using the stress-controlled rheometer AR-G2 (TA-instruments, Crawley, West-Sussex, UK) equipped with Pt-Ir du Noüy ring (Ø 13 mm). The formation of the surface film was followed during time and frequency dependent experiments with constant strain were done after the film had equilibrated. The strain dependent experiments with constant frequency were done after the film had equilibrated.

## 4.7 Matrix assisted laser desorption-ionization – time of flight mass spectrometry

Matrix assisted laser desorption-ionization – time of flight mass spectrometry (MALDI-TOF MS) is a method used to analyze proteins and peptides from complex samples such as mixtures of different proteins/peptides or even tissue specimens.[225,226]

The basic principle of MALDI-TOF is that proteins or peptides, dried on a target with light-absorbing matrix molecules, are vaporized by short laser pulses hitting the sample. The proteins/peptides are ionized by the laser pulse, usually with single charge ($z = 1$) and accelerated in an electrical field with in a vacuum. Ions with low mass to charge ration (m/z) are accelerated to higher velocity and hit the detector earlier than those with high m/z. The time of flight depends solely on the mass of the specimen because the separation is done in vacuum. The accurate mass of the specimen is determined by calibration with a known standard.[227,228]

MALDI-TOF MS (Bruker autoflex II MALDI-TOF) was used in Publication II to analyze the extent of AP modification with SFB and in Publication IV to verify the size of individual CBM and the glycosylation of DCBM.

## 4.8 General overview of genetic engineering, transformation to *Trichoderma reesei* and expression of double cellulose binding modules

*T. reesei* codon optimized synthetic genes encoding HFBI, DCBM-12, DCBM-24, and DCBM-48 flanking with compatible BsaI-sites were ordered from GenScript USA Inc. (NJ, USA) in BsaI-free pUC57 plasmids. Golden Gate cloning (GGC), was used for assembling the *T. reesei* transformation cassettes from six pieces into a

destination plasmid, which was based on the pMK-RQ. GCC is a cloning method based on the activity of BsaI restriction enzyme and it can be used to assemble multiple DNA pieces in a single reaction.[229,230] The assembled parts were (in 5'-3' order) 1) *cbh1* promoter, 2) HFBI coding sequence with signal sequence for secretion, 3) DCBM (12, 24 or 48) coding sequence, 4) *cbh1* terminator, 5) *hph* hygromycin resistance marker gene under the *gpdA* promoter for strain selection, and 6) a 3' flank sequence that together with the *cbh1* promoter was used to recombine the gene into the *cbh1* locus in *T. reesei*.

The ligation products of GGC were transformed into *E. coli* XL1-Blue cells and plasmids containing transformants were selected on kanamycin plates and further selected by blue-white screening for insert-containing clones. Correct assembly of the cassette was verified by restriction enzyme analysis and the correct protein coding sequence was verified by DNA sequencing. The transformation cassettes were cut from the vectors using PmeI and purified by agarose gel electrophoresis followed by gel extraction using dialysis.

Biolistic PDS-1000/He Particle Delivery System or gold particle bombardment (Bio-Rad, CA, USA) was used for the transformation of *T. reesei* M122 spores according to the instruction manual of Bio-Rad laboratories. The particle bombardment is based on the gold particles (in this work Ø 0.6 µm) delivering the gene of interest (the transformation cassette discussed above) in to the host cell with the aid of helium pressure[231]. Gold particles without DNA are used as a control. After transformation the spores were plated on potato dextrose agar (PD) plates with top-agar containing necessary salts and hygromycin for selection and grown in 28 °C. For transformant selection colonies were picked and streaked on PD-Triton plates containing hygromycin, grown in 28 °C, and then re-streaked. Insert containing transformants were identified by direct PCR (Phire® Plant Direct PCR kit, Finnzymes, F-130) using suitable oligonucleotide primers. Correct recombination localization into the *cbh1* locus was verified by the absence of an amplicon using *cbh1* specific primers.

The proteins were produced in 50 ml shake flask cultures (*T. reesei* minimal media, 4% lactose , 2% spent grain extract, 100 mM PIPPS (Piperazine-N,N′-bis(3-propanesulfonic Acid)) pH 5.5, 2.4 mM $MgSO_4$, 4.1 mM $CaCl_2$, 28°C, 7 days) and protein producing strains were identified by western blotting using rabbit anti-HFBI antibodies. The strains were then cultivated in 7 L bioreactors (50 vol-% spent grain extract, 60 $gL^{-1}$ lactose, 1 $gL^{-1}$ yeast extract, 4 $gL^{-1}$ $KH_2PO_4$, 2.8 $gL^{-1}$ $(NH_4)_2SO_4$, 0.6 $gL^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.8 $gL^{-1}$ $CaCl_2 \cdot 2H_2O$, 2 $mlL^{-1}$ trace solution). The culture supernatants were separated from the biomass by filtration. Protein expression levels were analyzed by RP-UPLC and were 0.2 $gL^{-1}$, 0.4 $gL^{-1}$, and 3.0 $gL^{-1}$ for HFBI-DCBM-12, -24, and -48, respectively. The proteins were purified RP-HPLC as described earlier followed by lyophilisation.

## 4.9 Calculation of binding constants and energies for DCBMs

For a given protein-substrate association, assuming Langmuir one site binding model applies and that the binding is reversible, binding is described by Equation (2).

$$B = \frac{[B_{max}] \times [Free]}{k_d \times [Free]} \quad (2),$$

where B is the specific binding, $[B_{max}]$ is the maximum specific binding, [Free] is the concentration of free protein and $k_d$ is the equilibrium binding constant that is analogous to the dissociation constant $K_d$. The $k_d$ -values can be calculated if reliable $B_{max}$-values are obtained. For CBM this number is very hard to determine because most methods are limited in giving accurate results at the high concentration range, where small error in the free protein concentration results in large errors in the bound protein. By derivation of Equation (2) we can obtain Equation (3).

$$B'_{[Free] \to 0} = \frac{[B_{max}]}{k_d} = K_r \quad (3),$$

where $K_r$ is the relative partition coefficient that describes the relation of the maximal binding and the equilibrium binding constant when the free protein concentration approaches zero. This is the initial slope of the isotherm function. This initial slope can be gained for all the proteins that have been studied in the thesis, mainly the five proteins in Publication IV. The Gibb's free energy, ΔG, of a given binding can be expressed by Equation (4).

$$\Delta G = -RTlnK_a = RTlnK_d = RTlnk_d \quad (4),$$

where R is the universal gas constant and T is the temperature in Kelvins. Now the $k_d$ can be expressed as the ratio of the partitioning coefficient, $K_r$, and maximum binding capacity, $B_{max}$, (Equation 3) and inserted in equation (4) where we get Equation (5). [232]

$$RTln\frac{B_{max}}{K_r} = RT\big(ln(B_{max}) - ln(K_r)\big) \quad (5)$$

This is useful since the initial slope for all proteins can be obtained and from the full set of data we can obtain $B_{max}$-values that are for DCBM-48 very good, and for DCBM-12 and DCBM-24 quite close to the actual value. For the single CBM the values are quite far from the actual values. An estimation of how the binding energy is affected by the $B_{max}$-value can be gained by overestimation of $B_{max}$, by multiplying the gained $B_{max}$ by two and three. By using those values, we can gain a range of

the free energy associated with the binding that shows the dependency of the energy to the maximum capacity. Figure 22 shows a graphical example of how this was done. In Publication IV we used this mathematical method to calculate the binding energies for DCBM-48 on NFC and BMCC, and to estimate the range of binding energies of the DCBM-12, DCBM-24, CBM-Cel7A and CBM- Cel6A.

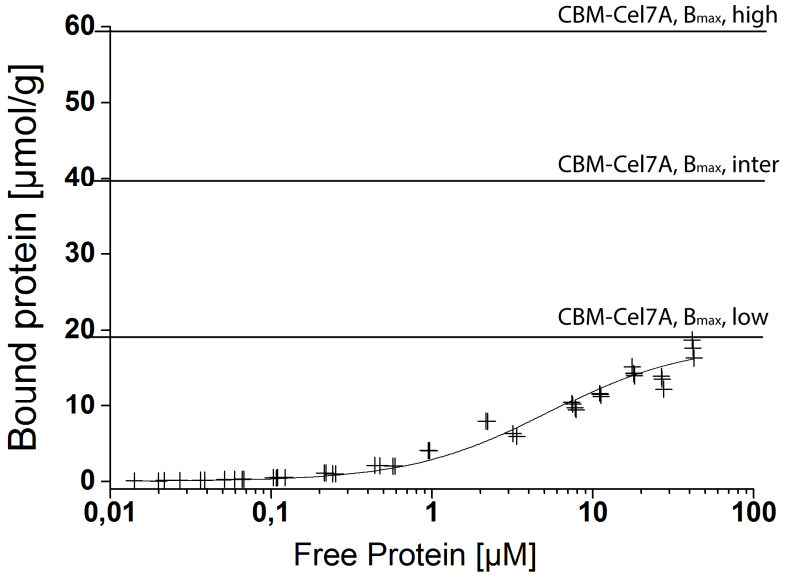


**Figure 22.** Graphical illustration of the $B_{max}$-values used to calculate the binding energies for the DCBM and CBM. The data is for CBM-Cel7A binding to NFC.

# 5. Conclusions and future prospective

Nanocellulose is a bio-based and renewable material with very interesting and promising properties. It has potential to be used in many high-tech applications where traditionally inorganic or fossil based materials are applied. The understanding of the materials properties and how it can be modified for different applications is a key question in nanocellulose research and application development.

In this work we took a biochemical approach in the modification and functionalization of nanocellulose surface. This approach was chosen for multiple reasons; firstly, biochemical reactions are performed in mild aqueous ambient conditions. Secondly, biology offers specific functions through proteins, which can be modified by genetic engineering to combine multiple proteins into one single recombinant protein. Genetic engineering can also be used to fine tune the protein properties or alter their function. Thirdly, enzymes can be used for very specific modification of materials, which is especially useful when it is important to preserve the original properties of the material. We show that biochemical methods are applicable to nanocellulose surface modification and functionalization and that these methods can be also used to gain new understanding of the materials properties.

By interaction and immobilization we showed that nanocellulose can be modified and functionalized by biochemical means giving new routes for use in surface and colloidal applications (Publications I & II). One critical limit, however, in applying these methods is the lack of understanding how nanocellulose as a substrate behaves. The aspects of this were shown by the role of hemicellulose in NFC matrix, and how the processing history affects the materials properties greatly (Publication III). Also the interaction studies of CBM and DCBM on NFC and BMCC show that NFC as a material is very different from BMCC (Publication IV). This shows that there is still a lot to be learned about cellulose as a substrate, even for the uses such as biofuels.

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|---|---|
| Title | **Biochemical modification and functionalization of nanocellulose surface** |
| Author(s) | Suvi Arola |
| Abstract | Cellulose is an abundant biopolymer found in many different organisms ranging from microbes to plants and animals. The homopolymer, composed of repeating glucose units, forms mechanically strong nanosized fibrils and rods. In plants cellulose forms macroscopic fibers, which are incorporated in the cell walls. Recently, it has been shown that cellulose fibers can be disintegrated into the fibrils and rods by different chemical treatments. These materials are called nanocellulose. Nanocellulose is a promising material to replace fossil based materials because it is renewable, biodegradable and abundant. It holds great potential in many applications due to its superior mechanical properties and large surface area. For most applications modification of nanocellulose surface is needed due to its tendency to aggregate by hydrogen bonding to adjacent cellulose surfaces.<br>In this thesis we took a biochemical approach on nanocellulose surface modification to achieve modified and functional materials. The advantages of this approach are that the reactions are done in mild aqueous ambient conditions and the amount of functionalities of biomolecules is broad. Four different approaches were chosen. First, genetically engineered cellulose binding proteins, were used to introduce amphiphilic nature to nanocellulose in order to create surface self-assembled nanocellulose films and to stabilize emulsions. This method was shown to be a good method for bringing new function to nanocellulose. (Publication I) Second, covalent coupling of enzymes directly onto modified nanocellulose surfaces provided a route for protein immobilization in bulk. Nanocellulose derivatives were shown to be well suited platforms for easy preparation of bioactive films. More over the film properties could be tuned depending on the properties of the derivative. (Publication II) Third, by modifying the nanocellulose surface with specific enzymes we could study the role of hemicellulose in nanocellulose fibril surface interactions. We showed that hemicellulose has an important role in nanofibrillated cellulose networks, yet its effects were different in aqueous and dry matrixes. (Publication III) Fourth, by modifying the specific function of cellulose binding protein via genetic engineering we showed how the binding properties can be altered and thus the functionalization properties can be tuned, and that the cellulose binding protein properties are substrate dependent. We also showed that nanocellulose as a model substrate in binding studies is a valuable tool for gaining new insight in protein binding behavior. (Publication IV)<br>In conclusion, we showed that biochemical methods are feasible in nanocellulose modification and functionalization to study intrinsic properties of nanocellulose and cellulose binding proteins but also for creating new functional materials. |
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| Nimeke | **Nanoselluloosapinnan biokemiallinen muokkaus ja funktionalisointi** |
| Tekijä(t) | Suvi Arola |
| Tiivistelmä | Selluloosa on laajalti luonnossa esiintyvä biopolymeeri, jota tuottavat kasvien ja mikrobien lisäksi jotkin eläinkunnan jäsenet. Tämä homopolymeeri, joka muodostuu toistuvista glukoosiyksiköistä, muodostaa mekaanisesti vahvoja nanokokoluokan fibrillejä ja tikkuja. Kasveissa selluloosa muodostaa mikro- ja makrokokoluokan kuituja, jotka ovat osana soluseinärakennetta. Hiljattain on pystytty näyttämään, että selluloosakuidut voidaan hajottaa fibrilleiksi ja tikuiksi erilaisin kemian menetelmin. Näitä materiaaleja kutsutaan nanoselluloosaksi. Nanoselluloosa on lupaava materiaali korvaamaan öljypohjaisia materiaaleja, koska se on uusiutuva, biohajoava ja helposti saatavilla oleva. Nanoselluloosa on myös potentiaalinen materiaali monilla eri teknologioiden aloilla suuren pinta-alansa ja ylivertaisten mekaanisten ominaisuuksiensa takia. Suurin osa sovelluksista vaatii nanoselluloosapinnan ominaisuuksien muokkaamista, koska nanoselluloosakuidut liittyvät helposti yhteen vetysidosten välityksellä.<br>Tässä väitöskirjassa on tutkittu biokemiallisten menetelmien soveltuvuutta nanoselluloosapintojen muokkaamisessa ja funktionalisoimisessa. Näiden menetelmien etuja ovat, että reaktiot tapahtuvat miedossa vedellisessä ympäristössä ja että biomolekyylien toiminnollisuuksien joukko on erittäin laaja. Valitsimme neljä erilaista lähestymistapaa. Ensinnä käytimme geneettisesti muokattuja selluloosaan sitoutuvia proteiineja tuomaan amfifiilisyyttä nanoselluloosan pinnalle. Näitä rakenteita käytettiin muodostamaan itsestään järjestäytyneitä pintoja nanoselluloosasta sekä stabiloimaan emulsioita. Tämän menetelmän näytettiin toimivan nanoselluloosan funktionalisoinnissa. (Osajulkaisu I) Toiseksi näytimme, että nanoselluloosan johdannaiset toimivat hyvin matriisina proteiinien suoralle kovalenttiselle kiinnittämiselle bulkissa. Nämä johdannaiset sopivat hyvin bioaktiivisten filmien valmistukseen. Lisäksi filmien ominaisuuksia voitiin muuttaa ja muokata nanoselluloosajohdannaisen ominaisuuksista riippuen. (Osajulkaisu II) Kolmanneksi tutkimme hemiselluloosan roolia nanoselluloosamatriisissa muokkaamalla nanoselluloosan pintaa spesifisillä entsyymeillä. Pystyimme näyttämään, että hemiselluloosalla on tärkeä rooli näissä verkostoissa, joskin se on erilainen kuivissa ja kosteissa systeemeissä. (Osajulkaisu III) Neljänneksi, muokkaamalla geneettisesti selluloosaan sitoutuvan proteiinin spesifiä toimintoa, näytimme, että pystymme vaikuttamaan sen sitoutumisominaisuuksiin ja näin mahdollisesti myös nanoselluloosamateriaalien toiminnollisuuteen. Näytimme myös, että selluloosaan sitoutuvan proteiinin toiminta on riippuvaista sen substraatista ja että nanoselluloosa on hyvä mallisubstraatti sitoutumiskokeissa, sillä se tuo uutta tietoa näiden proteiinien toiminnasta. (Osajulkaisu IV)<br>Yhteenvetona voi todeta, että erilaiset biokemialliset menetelmät soveltuvat nanoselluloosapinnan muokkaukseen ja funktionalisointiin, nanoselluloosan ja siihen sitoutuvien proteiinien luontaisten ominaisuuksien tutkimisessa, mutta myös uusien toiminnallisten materiaalien luomiseen. |
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## Biochemical modification and functionalization of nanocellulose surface

Cellulose is an abundant biopolymer found in many different organisms ranging from microbes to plants and animals. The homopolymer, composed of repeating glucose units, forms mechanically strong nanosized fibrils and rods. In plants cellulose forms macroscopic fibers, which are incorporated in the cell walls. Recently, it has been shown that cellulose fibers can be disintegrated into the fibrils and rods by different chemical treatments. These materials are called nanocellulose. Nanocellulose is a promising material to replace fossil based materials because it is renewable, biodegradable and abundant. It holds great potential in many applications due to its superior mechanical properties and large surface area. For most applications modification of nanocellulose surface is needed due to its tendency to aggregate by hydrogen bonding to adjacent cellulose surfaces.

In this thesis we took a biochemical approach on nanocellulose surface modification to achieve modified and functional materials.

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