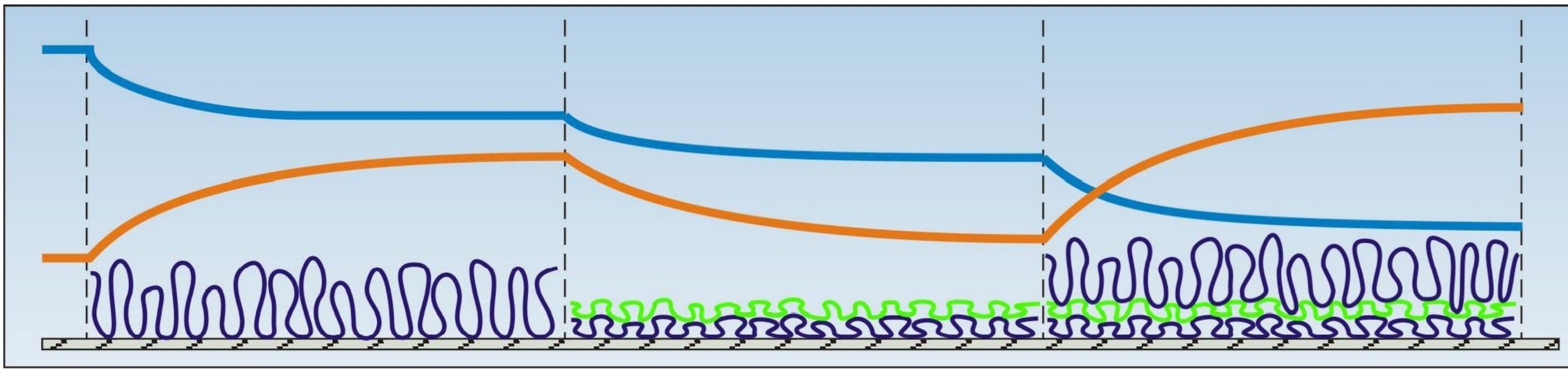
TKK Reports in Forest Products Technology, Series A5
Espoo 2008

ADSORPTION STUDIES OF POLYELECTROLYTES AND ENZYMES ON LIGNOCELLULOSIC MODEL SURFACES

Doctoral Thesis

Terhi Saarinen



TEKNILLINEN KORKEAKOULU
TEKNISKA HÖGSKOLAN
HELSINKI UNIVERSITY OF TECHNOLOGY
TECHNISCHE UNIVERSITÄT HELSINKI
UNIVERSITE DE TECHNOLOGIE D'HELSINKI

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ADSORPTION STUDIES OF POLYELECTROLYTES AND ENZYMES ON LIGNOCELLULOSIC MODEL SURFACES

Doctoral thesis

Terhi Saarinen

Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Faculty of Chemistry and Materials Sciences for public examination and debate in Auditorium AS1 at Helsinki University of Technology (Espoo, Finland) on the 12th of December, 2008, at 12 noon.

Helsinki University of Technology
Faculty of Chemistry and Materials Sciences
Department of Forest Products Technology

Teknillinen korkeakoulu
Kemian ja materiaalitieteiden tiedekunta
Puunjalostustekniikan laitos

Distribution:
Helsinki University of Technology
Faculty of Chemistry and Materials Sciences
Department of Forest Products Technology
P.O. Box 6300
FIN-02015 TKK

ISBN 978-951-22-9653-8 (printed)
ISSN 1797-4496
ISBN 978-951-22-9654-5 (PDF)
ISSN 1797-5093
URL: http://lib.tkk.fi/Diss/2008/isbn9789512296545/

Picaset Oy
Helsinki 2008

ABSTRACT OF DOCTORAL DISSERTATION	HELSINKI UNIVERSITY OF TECHNOLOGY P.O. BOX 1000, FI-02015 TKK http://www.tkk.fi

Author Terhi Saarinen

Name of the dissertation
Adsorption studies of polyelectrolytes and enzymes on lignocellulosic model surfaces

Manuscript submitted 09.09.2008	Manuscript revised 10.11.2008

Date of the defence 12.12.2008

☐ Monograph	☒ Article dissertation (summary + original articles)

Faculty	Faculty of Chemistry and Materials Sciences
Department	Department of Forest Products Technology
Field of research	Chemistry of Forest Products Technology
Opponent(s)	Prof. Maija Tenkanen
Supervisor	Prof. Janne Laine
Instructor	Ph.D., Docent Monika Österberg

Abstract

This thesis presents fundamental studies on the adsorption of polyelectrolytes and enzymes on solid surfaces. The overall objective of the research was to clarify the adsorption phenomena of polyelectrolytes and enzymes taking place in papermaking. The adsorption experiments were made with a quartz crystal microbalance with dissipation, QCM-D, and the enzyme-modified surfaces were characterised by atomic force microscopy, AFM.

In order to study the interactions taking place at a molecular level, model surfaces of different pulp components are needed. Therefore, at first the preparation and characterisation of cellulose and lignin model films for QCM-D studies were evaluated. The adsorption of single cationic polyelectrolytes and of the polyelectrolyte multilayers and complexes on silica and cellulose surfaces was studied. The low-charge cationic polyelectrolytes adsorbed more on silica while the adsorption of high-charge polyelectrolytes was higher on cellulose. At the beginning of the adsorption the polyelectrolyte layers were viscous and more dissipative on cellulose and when the adsorption proceeded the layers became more rigid, in contrast to the behaviour on silica. The low-charge complexes (PECs) were more dissipative and viscous than those of high-charge PECs. The multilayers and complexes formed by low-charge polyacrylamides formed rather thick layers on both surfaces, unlike the high-charge polyelectrolytes which formed flat and thin layers with both addition techniques. By using polyelectrolyte complexes instead of the multilayering technique, thick and dissipative layers with a lower amount of polyelectrolyte can be formed.

The modification of lignocellulosic model surfaces with laccases and further the adsorption of anionic ferulic acid on laccase-activated lignin were also examined by QCM-D. Both enzymes, *Trametes hirsuta* and *Melanocarpus albomyces* laccases adsorbed on cellulose and lignin surfaces. The adsorbed amount of *M. albomyces* laccase was higher than that of *T. hirsuta* laccase and the adsorption of *M. albomyces* laccase was strongly dependent on pH on the lignin surface. Stable ferulic acid adsorption was found on laccase-activated lignin but the mode of action of laccases was different when laccase and ferulic acid were added simultaneously on lignin.

Keywords adsorption, AFM, cellulose, laccase, lignin, polyelectrolyte, polyelectrolyte complex, polyelectrolyte multilayer, QCM-D

ISBN (printed)	978-951-22-9653-8	ISSN (printed)	1797-4496
ISBN (pdf)	978-951-22-9654-5	ISSN (pdf)	1797-5093
Language	english	Number of pages	93 p.
Publisher	Helsinki University of Technology		
Print distribution	Helsinki University of Technology		

☒ The dissertation can be read at http://lib.tkk.fi/Diss/

VÄITÖSKIRJAN TIIVISTELMÄ

TEKNILLINEN KORKEAKOULU
PL 1000, 02015 TKK
http://www.tkk.fi

Tekijä Terhi Saarinen

Väitöskirjan nimi
Polyelektrolyyttien ja entsyymien adsorptiotutkimus lignoselluloosamallipinnoilla

Käsikirjoituksen päivämäärä 09.09.2008

Korjatun käsikirjoituksen päivämäärä 10.11.2008

Väitöstilaisuuden ajankohta 12.12.2008

☐ Monografia

☒ Yhdistelmäväitöskirja (yhteenveto + erillisartikkelit)

Tiedekunta	Kemian ja materiaalitieteiden tiedekunta
Laitos	Puunjalostustekniikan laitos
Tutkimusala	Puunjalostuksen kemia
Vastaväittäjä(t)	Prof. Maija Tenkanen
Työn valvoja	Prof. Janne Laine
Työn ohjaaja	FT, Dosentti Monika Österberg

Tiivistelmä

Työssä esitetään polyelektrolyyttien ja entsyymien adsorptiotutkimusta kiinteille mallipinnoille. Tavoitteena oli selvittää paperinvalmistuksessa esiintyvien polyelektrolyyttien ja entsyymien adsorptioilmiöitä. Adsorptiot tehtiin kvartsikidemikrovaa'alla (QCM-D) ja entsyymimodifioituja pintoja tarkasteltiin atomivoimamikroskoopilla (AFM).

Puukuitujen eri komponenteista valmistettuja mallipintoja tarvitaan, jotta pystytään tutkimaan paperinvalmistuksessa esiintyviä vuorovaikutuksia molekyylitasolla. Tämän takia työn alussa kehitettiin ja karakterisoitiin selluloosa- ja ligniinimallipintojen soveltuvuus QCM-D -1aitteeseen. Aluksi tutkittiin yksittäisten kationisten polyelektrolyyttien ja monikomponenttisysteemien adsorptiota piidioksidi- ja selluloosapinnoille. Alhaisesti varautunut kationinen polyelektrolyytti adsorboitui enemmän piidioksidille kuin korkeavarauksinen polyelektrolyytti, jonka adsorptio oli suurempi selluloosalla. Adsorption alussa polyelektrolyyttikerrokset olivat viskoottisia ja dissipatiivisia selluloosalla ja adsorption edetessä kerrokset muuttuivat tiiviimmiksi päinvastoin kuin piidioksidipinnalla. Alhaisesti varautuneet polyelektrolyyttikompleksit olivat viskoottisempia ja löyhempiä kuin korkeasti varautuneet kompleksit. Alhaisesti varautuneista polyakryyliamideista muodostettujen monikerrosrakenteiden ja kompleksien adsorptio ja kerrospaksuus olivat suuria molemmilla pinnoilla päinvastoin kuin korkeasti varautuneet polyelektrolyytit, jotka muodostivat litteitä ja ohuita kerroksia kummallakin lisäystavalla. Polyelektrolyyttikompleksit muodostivat paksuja ja löyhiä kerroksia pienemmällä polyelektrolyyttimäärällä monikerrosrakenteisiin verrattuna.

Lignoselluloosamallipintoja modifioitiin myös lakkaaseilla ja tutkittiin ferulahapon adsorptiota lakkaasiaktivoidulle ligniinille QCM-D:llä. Molemmat entsyymit, *Trametes hirsuta* ja *Melanocarpus albomyces* lakkaasi, adsorboituivat selluloosalle ja ligniinille. *T. hirsuta* lakkaasin adsorptio oli alhaisempi kuin *M. albomyces* lakkaasin. *M. albomyces* lakkaasin adsorptio oli voimakkaasti pH-riippuvainen ligniinillä. Ferulahappo adsorboitui pysyvästi lakkaasimodifioituun ligniinipintaan. Lisättäessä lakkaasi ja ferulahappo samanaikaisesti vain *T. hirsuta* lakkaasi pystyi aktivoimaan sekä ferulahapon että ligniinin ja kiinnittämään ferulahapon irreversiibelisti ligniinipintaan.

Asiasanat adsorptio, AFM, lakkaasi, ligniini, polyelektrolyytti, polyelektrolyyttikompleksi, polyelektrolyyttimonikerrosrakenne, QCM-D, selluloosa

ISBN (painettu) 978-951-22-9653-8
ISSN (painettu) 1797-4496
ISBN (pdf) 978-951-22-9654-5
ISSN (pdf) 1797-5093
Kieli englanti
Sivumäärä 93 s.
Julkaisija Teknillinen korkeakoulu
Painetun väitöskirjan jakelu Teknillinen korkeakoulu

☒ Luettavissa verkossa osoitteessa http://lib.tkk.fi/Diss/

PREFACE

This work was carried out at the Department of Forest Products Technology, Helsinki University of Technology between 2001 and 2008.

The financiers of the research, the National Technology Agency (TEKES), and the industrial companies Stora Enso Oyj, M-real Oyj, UPM Kymmene Corporation, Kemira Oyj, Ciba Specialty Chemicals, Metsä-Botnia and Metso Paper, are gratefully acknowledged for their contribution.

First and foremost I would like to thank my supervisor, Professor Janne Laine, for giving me the opportunity to complete this thesis and for guiding me through the research. His scientific expertise, encouragement, and patience were invaluable to me during these years. I am also deeply grateful to Professor Emeritus Per Stenius for giving me the chance to start working in his laboratory. Thanks are also due to my instructor Docent Monika Österberg for sharing her extensive knowledge in physical chemistry as well as for supervising my scientific writing. Dr. Susanna Holappa is gratefully acknowledged for her valuable comments regarding the laccase manuscript.

In addition, I would like to thank the staff of Laboratory of Forest Products Chemistry for the great and encouraging working atmosphere. The laboratory work performed by Ritva Kivelä, Marja Kärkkäinen and Anu Anttila was invaluable. My special thanks go Aila Rahkola for preparing the Langmuir-Schaefer cellulose surfaces. Kati Mäenpää is thanked for her help with the literature acquisition.

I also want to thank Anna Suurnäkki, Stina Grönqvist and Martina Andberg at VTT Technical Research Centre of Finland for the fruitful and pleasant collaboration.

Leena-Sisko Johansson and Joseph Campbell are gratefully acknowledged for performing the ESCA measurements and for giving valuable comments on the results.

Olof Andersson and Joseph Campbell are thanked for the linguistic revision of the manuscript.

Furthermore, I want to thank my present and former colleagues Monika, Susanna, Taina, Tiina, Susanna, Minna, Hanna, Anna-Maija, Krista, Katri, Mari and Kati for their friendship and enjoyable discussions during lunch breaks.

My deepest gratitude goes to my husband Ari and sons Aleksi and Oskari for their endless love and support. I also want to thank all my relatives and friends for being there for me during these years.

Espoo, 29th September 2008
Terhi Saarinen

LIST OF PUBLICATIONS

This thesis is based on the results presented in five publications, which are referred to as Roman numerals in the text. Some other results are also included regarding the ageing of polyacrylamide solutions.

Paper I

Tammelin, T., Saarinen, T., Österberg, M. and Laine, J. (2006). Preparation of Langmuir/Blodgett-cellulose surfaces by using horizontal dipping procedure. Application for polyelectrolyte adsorption studies performed with QCM-D. *Cellulose* 13(5), 519-535.

Paper II

Saarinen, T., Österberg, M. and Laine, J. (2009). Properties of cationic polyelectrolyte layers adsorbed on silica and cellulose surfaces studied by QCM-D – Effect of polyelectrolyte charge density and molecular weight. *Journal of Dispersion Science and Technology* 30(6), accepted.

Paper III

Saarinen, T., Österberg, M. and Laine, J. (2008). Adsorption of polyelectrolyte multilayers and complexes on silica and cellulose surfaces studied by QCM-D. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 330(2-3), 134-142.

Paper IV

Saarinen, T., Orelma, H., Grönqvist, S., Andberg, M., Holappa, S. and Laine, J. (2009). Adsorption of laccases on cellulose and lignin studied by QCM-D and AFM. *BioResources* 4(1), 94-110.

Paper V

Saarinen, T., Suurnäkki, A., Österberg, M. and Laine, J. (2008). Modification of lignin with laccases for the adsorption of anionic ferulic acid studied by QCM-D and AFM. *Holzforschung,* accepted.

AUTHOR’S CONTRIBUTION

Paper I

Terhi Saarinen defined the research plan with co-authors, performed mainly the QCM-D experiments, analysed the experimental work and wrote the manuscript with the co-authors.

Papers II-III

Terhi Saarinen defined the research plan with co-authors, performed the experimental work related to model surface preparations, QCM-D measurements, analysed the results, performed the modellings and wrote the manuscripts with the co-authors.

Papers IV-V

Terhi Saarinen defined the research plan with co-authors, performed the QCM-D experiments, analysed the experimental work and wrote the manuscripts with the co-authors.

LIST OF SYMBOLS AND ABBREVIATIONS

AFM	atomic force microscopy
A-PAM	anionic polyacrylamide
ρ_{Voigt}	assumed density of the adsorbed layer used in Voigt model
C	sensitivity constant of QCM-D instrument
CD	charge density
C-PAM	cationic polyacrylamide
$\Delta\mathrm{D}$	change in dissipation
$d_{Sauerbrey}$	thickness of the adsorbed layer obtained from Sauerbrey equation
d_{Voigt}	thickness of the adsorbed layer obtained from Voigt model
E_{diss}	total dissipated energy during one oscillation cycle
E_{stor}	total energy stored in the oscillation
ESCA	electron spectroscopy for chemical analysis
FA	ferulic acid, 4-hydroxy-3-methoxycinnamic acid
f_0	fundamental resonance frequency
Δf	change in frequency
η_{dyn}	dynamic viscosity
η_f	fluid viscosity
LS	Langmuir-Schaefer dipping technique
$\Delta m_{Sauerbrey}$	adsorbed mass according to Sauerbrey equation
Δm_{Voigt}	adsorbed mass according to Voigt model
MaL	*Melanocarpus albomyces* laccase
Mw	molecular weight
MWL	milled wood lignin
n	number of overtone
ρ_f	fluid density
PDADMAC	polydimethyldiallylammonium chloride
PE	polyelectrolyte
PEC	polyelectrolyte complex
PEM	polyelectrolyte multilayer
PESNa	sodium polyethylene sulphonate
PS	polystyrene

QCM-D	quartz crystal microbalance with dissipation
SiO_2	silicon dioxide
ThL	*Trametes hirsuta* laccase
TMSC	trimethylsilyl cellulose

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AUTHOR’S CONTRIBUTION

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1 INTRODUCTION

Adsorption is a phenomenon in which a gas or liquid solute adheres on a solid surface. As a consequence, an increase in concentration of the solute in the interfacial region takes place. At the molecular level, adsorption is due to attractive interactions between the surface and the adsorbed molecules. Adsorption mechanisms can be divided into physical, chemical and electrostatic adsorption (Fleer et al. 1993). Weak intermolecular forces, such as Van der Waals forces, provide the driving force for physical adsorption, while chemical bonds between the compound and the solid surface are formed during chemical adsorption. The electrostatic adsorption involves adsorption of ions through Coulombic forces, also called ion exchange. The reduction in solute concentration at the interface is called negative adsorption or depletion.

In papermaking many different chemical additives are used in order to improve the efficiency of the formation process, e.g. retention and dewatering and to improve sheet properties (Roberts 1996). The additives adsorb on different solid surfaces, like cellulosic fibres, fines and fillers (Laine 2007). The adsorption of polyelectrolytes on fibres and fines is an essential first step in assisting their retention in the fibre web during sheet formation (Roberts 1996). Because of different type of raw material and various types of additives, it is imperative to understand the complex interactions between the different substances in the paper matrix.

At present, stagnation point adsorption reflectometry (SPAR) (Wågberg and Nygren 1999, Geffroy et al. 2000, Wågberg et al. 2004,), ellipsometry (Shubin and Linse 1995, Ödberg et al. 1995, Stemme et al. 1999, Samoshina et al. 2003) and quartz crystal microbalance with dissipation (QCM-D) (Munro and Frank 2004, Tammelin et al. 2004, Notley et al. 2005, Åsberg et al. 2005) are the most commonly used methods to study the polyelectrolyte adsorption at a molecular level and the thickness of adsorbed polyelectrolyte layers. When comparing the results obtained with these different methods it is important to remember that they measure different phenomena, which consequently affects the results. SPAR determines only the adsorbed amount, whereas ellipsometry provides both the average optical layer thickness as well as the adsorbed mass. In contrast to the optical techniques, which are not sensitive to water associated with the adsorbed layer, QCM-D takes into account the total mass, including bound water. The viscoelastic properties of the adsorbed layer can also be studied using QCM-D.

2 AIMS AND OUTLINE OF THE STUDY

This thesis presents fundamental studies on the adsorption phenomena of polyelectrolytes and enzymes on solid surfaces. The overall objective was to clarify the adsorption phenomenon of polyelectrolytes and enzymes taking place at a molecular level in papermaking. In addition to adsorbed amount, the properties and structure of the adsorbed layers of single polycations, polyelectrolyte multicomponents and enzymes affect the function of papermaking additives and further the properties of the final paper. Adsorption of oxidative enzymes on wood fibres enables the attachment of targeted functionalised groups, such as ferulic acid to fibres via enzymatic or chemo-enzymatic methods and further improves most of the desired properties of the fibres.

The experiments concentrated on adsorption studies conducted with a quartz crystal microbalance with dissipation, QCM-D. By using QCM-D the adsorption phenomenon, the factors affecting the adsorption and the conformation and viscoelastic properties of the adsorbed layers can be considered. Atomic force microscopy, AFM was also used for the characterisation of the enzyme-modified substrates. In order to study the interactions taking place at a molecular level in papermaking conditions, model surfaces of different pulp components are needed. To this end, the preparation and characterisation of Langmuir-Schaefer cellulose film (Paper I) as well as the spin-coated lignin substrate for QCM-D studies were evaluated.

The adsorption experiments can be divided into two parts. In the first part, which is covered by Papers II and III, the adsorption of single cationic polyelectrolytes as well as the polyelectrolyte multicomponent systems on model silica and cellulose surfaces was studied.

In the latter section (Papers IV and V) the modification of lignin and cellulose with laccases and further the adsorption of ferulic acid on laccase-activated lignin were considered.

The adsorption of single polyelectrolytes on oppositely charged solid surfaces has been studied by many researchers but the structure and properties of the adsorbed layers of polyelectrolytes on cellulose are still not fully understood (Paper II). The interactions between cationic and anionic polyelectrolytes, either forming multilayers (PEMs) or complexes (PECs, Paper III) were studied. PEMs have been studied in some detail by other researchers but only a few studies are concerned with the adsorption phenomenon of PECs or the properties of the adsorbed PEC layer on a solid surface. Finally, the layer properties of single polyelectrolytes, PEMs and PECs were compared.

In Papers IV and V the modification of cellulose and lignin model substrates by laccases was investigated. In lignin-rich pulps laccases can be used to activate the surface lignin by radicalisation. In lignin activation the laccases could also interact with other wood fibre components and the affinity of the laccases e.g. to cellulose is not yet clear. Therefore, in Paper IV the attachment of two laccases to cellulose and lignin was studied by QCM-D and the morphology of the treated surfaces by AFM. After enzymatic radicalisation, specific chemical components can be bound to fibres to tailor the fibre properties. In Paper V the chemo-enzymatic adsorption of laccase and ferulic acid, added either sequentially or as a complex, was studied.

The first main aim of this thesis, which concentrated to use QCM-D as a research tool, was to develop lignocellulosic model surfaces for QCM-D studies. By using these model surfaces the second objective was to examine and understand the adsorption behaviour of different type of polymeric and enzymatic systems. The third aim was to utilise the obtained information on the adsorption of enzymes on lignocellulosic surfaces to understand chemo-enzymatic adsorption of ferulic acid at a molecular level.

3 BACKGROUND

3.1 Polymers in solution

Polyelectrolytes are polymers carrying ionisable groups. In aqueous solutions these groups can dissociate, leaving charges on polymer chains and releasing counterions in solution (Fleer et al. 1993, Jönsson et al. 1998, Dobrynin and Rubinstein 2005). Polyelectrolytes can be classified according to the nature of the ionic groups as polyacids, polybases and polyampholytes. The properties of the polyelectrolytes in solution depend on the fraction of dissociated ionic groups, solvent, pH and salt concentration.

In solution the uncharged polymer behaves like a long mobile chain which is found to be flexible. The conformation changes randomly due to thermal motion. If no restrictions exist, the size of the polymer coil depends on the solvent and on the molecular weight of the polyelectrolytes. Some restrictions, such as chain structure and intramolecular interactions can, however, restrict the mobility of the monomer units. The conformation of the charged polyelectrolyte depends mainly on the electrostatic interactions between the charged groups of the polyelectrolyte and the ionic strength. High charged polyelectrolyte generates an increased osmotic pressure, which causes the chains to adopt a more expanded, rigid and rod-like conformation. The expansion of the polyelectrolyte decreases when the charge density of the polyelectrolyte decreases or alternatively the ionic strength increases. (Jönsson et al. 1998).

A simple measure of the polyelectrolyte coil is the mean distance between the polymer ends, R_m. Another measure of the polymer chain size is the radius of gyration, R_g, which describes the average distance of polymer segments from the centre of mass of the molecule. The radius of gyration depends on the molecular weight of the polyelectrolyte and the solubility of the solution. Solubility describes the relative strength of the polymer segment/segment and segment/solution interactions. A high affinity of the polymer for the solvent, i.e., a good solvent, leads to an osmotically swollen coil with a maximum radius of gyration. When the polymer affinity to the solution decreases a more compact molecule structure is formed. (Eisenriegler 1993, Fleer et al. 1993, Jönsson et al. 1998).

3.2 Polyelectrolyte adsorption

Adsorption of charged polymers on charged surfaces has been the subject of extensive theoretical and experimental studies for the last four decades (Wågberg 2000, Dobrynin and Rubinstein 2005). A significant part of the theoretical modelling was carried out utilising the self-consistent-field theory (Scheutjens and Fleer 1979, Scheutjens and Fleer 1980, Fleer et al. 1993). The extension of the self-consistent-field theory, considering the polymer charge density distribution, was applied by Van der Schee and Lyklema (1984) and Evers et al. (1986) and a further extension of the Van der Schee and Lyklema theory (1984) to the case of weak polyelectrolytes was done by Böhmer et al. (1990).

In general, the polyelectrolyte will attempt to neutralise all opposite charges on the surface. The adsorption of polyelectrolyte at low ionic strength on an oppositely charged surface can be regarded as an ion-exchange process. Several factors affect the adsorption of polyelectrolytes on solid surfaces (Fleer et al. 1993, Tiberg et al. 2001):

i. specific attractive interactions between the polyelectrolyte and the surface such as electrostatic and non-electrostatic interactions,
ii. gain in entropy when a large number of small solvent molecules are released from surface upon adsorption of a large polyions. Thermodynamically the entropic force favours adsorption.
iii. polyelectrolyte-solvent interactions. The solvent either favours or opposes the adsorption. In a good solvent polyelectrolytes interact rather with solvent, whereas in a poor solvent the polyelectrolytes tend to adsorb on surfaces.

3.2.1 Effect of polyelectrolyte properties on adsorption

The properties of the polyelectrolyte itself strongly affect the polyelectrolyte adsorption and further the structure of the adsorbed layer. For charged polyelectrolytes, electrostatic interactions affect both polyelectrolyte-solvent and polyelectrolyte-surface interactions, as well as interactions between the charged groups within one polyelectrolyte chain. Polyelectrolytes with a low charge density may adopt a coiled conformation in solution and upon adsorption a conformation with loops and tails is formed at the solid surfaces. Increasing the charge density of the polyelectrolyte results in a strong repulsion between the charged segments and a more expanded conformation. These factors promote a thinner and a more flat conformation at solid surfaces that furthermore prevents more polyelectrolytes to adsorb. Therefore, less polyelectrolyte is needed for neutralisation of the surface charge, indicating a decrease in the adsorbed amount (Figure 3.1, Evers et al. 1986, van de Steeg et al. 1992, Shubin and Linse 1997, Rojas et al. 2002). The effect of charge density of the polyelectrolyte on the adsorption on cellulose fibres has generally been found to be the same as for the polyelectrolyte adsorption on a smooth surface, i.e. the lower the charge density the higher the level of adsorption (Roberts 1996, Wågberg 2000).

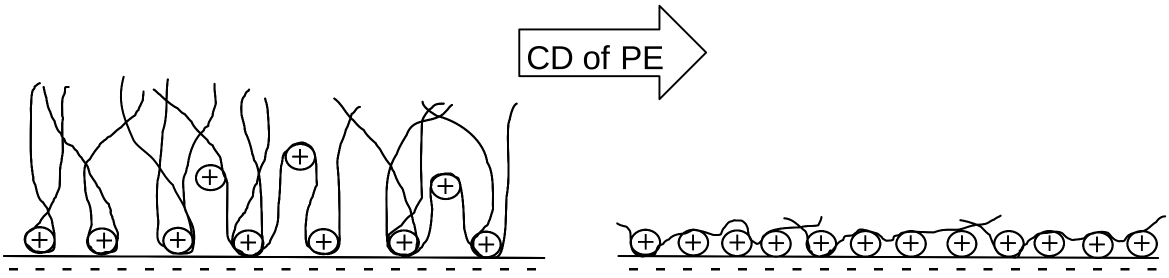


Figure 3.1. *Schematic drawing of the adsorption of cationic polyelectrolyte on high-charge anionic substrate. The charge density (CD) of the polyelectrolyte increases from left to right.*

The pH of the polyelectrolyte solution can also affect the properties of the polyelectrolyte and further the adsorption phenomenon. Strong polyelectrolytes dissociate completely in polar media and are pH-independent. The degree of dissociation of weak polyelectrolytes is related to the pH and they are partially dissociated at intermediate pHs (pH 2-10). Thus, weak polyelectrolytes are not fully charged in solution and the charge density of the polyelectrolytes can be modified by changing the solution's pH.

The molecular weight of the polyelectrolyte also affects the adsorption of polyelectrolytes on solid surfaces. According to theory (Böhmer et al. 1990, van de Steeg et al. 1992, Fleer et al. 1993, Tanaka et al. 1997), the adsorption remains almost constant or increases with an increase in the molecular weight of the polyelectrolyte on a smooth and high-charge surface. On a porous surface the effect is expected to be the opposite since smaller polyelectrolyte molecules can penetrate into the surface. An increase in the polyelectrolyte adsorption has been found on different pulps upon decreasing the molecular weight of the polyelectrolyte since more surfaces will be available for the smaller polymer (Roberts 1996, Wågberg 2000, Laine et al. 2007).

3.2.2 Effect of ionic strength on adsorption

The ionic strength of the solution is important and can either increase or decrease the adsorption, depending on the conditions (van de Steeg et al. 1992). Electrolyte affects the adsorption of polyelectrolytes via four mechanisms (Shubin and Linse 1995):

i. screening of electrostatic polyelectrolyte-surface attraction,
ii. screening of electrostatic repulsion between charged polymer segments,
iii. competition between polymer segments and small ions for the space near the surface,
iv. specific adsorption of counterions to the surface.

An increase in salt concentration decreases the electrostatic repulsion between charged polyelectrolyte segments (van de Steeg et al. 1992, Shubin and Linse 1995, Wågberg 2000). The screened polyelectrolytes adopt a more coiled conformation, meaning that more polyelectrolytes fit on the surface. Thus, the adsorbed amount increases upon increasing the ionic strength if the specific non-electrostatic interactions between the surface and polyelectrolyte are strong enough to keep the screened polyelectrolyte on the surface. This is typical for high-charge polyelectrolytes.

Salt can, however, screen not only the repulsion between charged polyelectrolyte segments but also the segment-surface attraction (van de Steeg et al. 1992, Shubin and Linse 1995, Wågberg 2000). If the adsorption is dominated by this electrostatic attraction, the adsorption decreases with an increase in salt concentration. This is typical for low-charge polyelectrolytes. A maximum in the adsorbed amount can also occur if the adsorption first increases upon increasing the salt concentration due to screening of repulsion between the charged segments. Further increasing the ionic strength the adsorption starts to decrease since the electrostatic attraction between the polyelectrolyte and surface becomes screened (Lindström and Wågberg 1983). At very high ionic strength, when salt has screened all the electrostatic interactions, the polyelectrolytes behave like uncharged polymers and the adsorption is possible only if there exists non-electrostatic forces.

3.2.3 Effect of substrate on adsorption

The adsorption of polyelectrolytes on uncharged surface differs obviously from the adsorption on charged surface since there is no electrostatic driving force for adsorption. Hence, the non-electrostatic polyelectrolyte-surface affinity must be sufficiently high for any adsorption to occur. The adsorbed amount of polyelectrolyte is expected to be small at low ionic strengths and the conformation of the adsorbed polyelectrolyte to be very flat since it is only near the surface where the non-electrostatic interactions between the surface and the polyelectrolyte chains affect (Fleer et al. 1993, Claesson et al. 2005). Polyelectrolyte adsorption on oppositely charged surface is mainly result from the electrostatic attraction between the surface charges and the charges in the polyelectrolyte chains, including the entropic gain due to the release of counterions (Fleer et al. 1993, Claesson et al. 2005). The adsorbed amount of polyelectrolyte is found to increase upon increasing the surface charge density (van de Steeg et al. 1992).

The surface roughness and porosity have an influence on the adsorbed amount of polyelectrolyte. On the other hand, properties of the solution present in the adsorption process, such as pH and salt concentration, could also affect the properties of the surface and further the adsorption of polyelectrolytes.

The adsorption of polyelectrolytes on pulp fibres has been found to correspond well to the above presented theoretical principles although the properties and structure of the adsorbed layers on lignocellulosic surfaces is not yet fully understood. Many experiments have shown that the adsorption of polyelectrolytes on cellulose fibres can be affected by addition of salt (Lindström and Wågberg 1983, Wågberg 2000, Laine 2007). Typically the adsorption is initially increased by increased ionic strength and then decreased when the ionic strength is further increased. Different pulps have been shown to have very different surface charge content, depending on the manufacturing method and wood species used (Laine 2007). Higher surface charge is reflected in higher consumption of cationic polyelectrolytes such as dry-strength and retention chemicals (Roberts 1996). The fines have a much larger surface area than the fibres so the clearly higher amount of cationic polyelectrolyte is adsorbed on the surface of fines (Wågberg 2000, Laine 2007). Furthermore, e.g. increased pH of the solution increases the adsorption of polyelectrolytes on cellulosic fibres since at higher pH values more polyelectrolyte is needed to neutralise the increased dissociation of the carboxylic groups on the fibres (Lindström and Wågberg 1983).

3.3 Multicomponent systems

In this chapter the polyelectrolyte multicomponent systems, including polyelectrolyte multilayers (PEMs) and polyelectrolyte complexes (PECs), are described. The PEMs are constructed by sequential deposition of oppositely charged polyelectrolytes on solid surfaces while premixing the oppositely charged polyelectrolytes generates PECs. Obviously some complexation between poyanions and polycations may also occur in the PEMs (Picart et al. 2002, Lavalle et al. 2004, Hubbe 2005, Hubbe et al. 2005, Heermann et al. 2006), however in the following these observations are still discussed together with polyelectrolyte multilayering. Only systems were the complexation occurs in solution prior to adsorption are treated as pure PECs.

3.3.1 Polyelectrolyte multilayers

The alternate deposition of polycations and polyanions on solid surfaces leads to the formation of films called polyelectrolyte multilayers (PEMs, Figure 3.2). While building up the PEMs, each step follows the fundamental principles of polyelectrolyte adsorption on solid surfaces. The electrostatic attraction between oppositely charged molecules and charge reversal at each step is the basis for the formation of PEMs (Decher 1997). Thus, the electrostatically driven multilayer formation needs a minimum charge density of both surface and the oppositely charged polyelectrolytes (Schoeler et al. 2002, Voigt et al. 2003). Increasing the charge density of the polyelectrolytes (above the critical charge density) leads to a stronger electrostatic repulsion between the polyelectrolyte chains and thus, a flatter and thinner conformation on the surface. With increasing polyelectrolyte concentrations or salt concentration the charges along the polyelectrolyte chain become more screened which leads to an enhanced coiling of the chains and an increase in adsorbed amount and layer thickness.

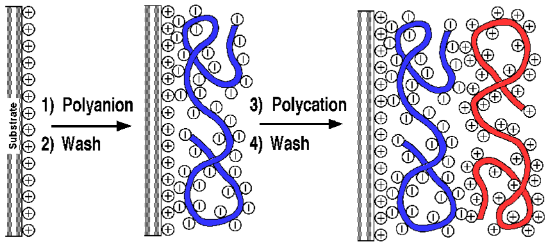


Figure 3.2 *Schematic representation of multilayer formation by alternating polyelectrolyte deposition, starting with a positively charged substrate. Counterions are omitted for clarity (Decher 1997).*

The build-up of PEMs has been characterised as growing either linearly or exponentially. In an exponentially growing model the multilayer thickness increases exponentially with the number of deposited layers (Picart et al. 2002, Lavalle et al. 2004). The polycations diffuse into the film during the addition step but when rinsing they diffuse outward of the film. Addition of polyanions achieves a complexation with the polycations in the upper layer of the film. These complexes form the new upper layer of the film. The amount of polycation diffusing into the interface is proportional to the film thickness. In the case where no diffusion of the polyelectrolytes within the multilayer occurs the film thickness grows linearly (Hoogeveen et al. 1996b). Thus, the structure of the PEM is highly dependent on the composition and conformation of the polyelectrolyte in the outermost layer (Lösche et al. 1998, Eriksson et al. 2005, Notley et al. 2005).

The build-up of PEMs by oppositely charged polyelectrolytes is a versatile method enabling the construction of ultrathin films with well-defined thickness, composition and chemical functionalities. The main benefits of the PEM coatings are the possibility to combine the properties of two or more polyelectrolytes in one system to conformably coat objects. PEM treatment is already used in several applications, such as in sensor technology (Sun et al.

1996), in light-emitting thin films (Fou et al. 1996) and in capsules for drug delivery (Qiu et al. 2001). The modification of adhesion properties of surfaces by PEM treatment also seems to be promising, e.g. in enhancing the wettability and strength properties of wood fibres (Wågberg et al. 2002, Hubbe et al. 2003, Lingström et al. 2006, Vainio et al. 2006, Enarsson and Wågberg 2007). The greatest strength gains were achieved when the amount of the first additive was many times greater than the adsorption capacity of the fibres. In this case there is excess cationic polyelectrolyte in solution and polyelectrolyte complexes are formed between the cationic polyelectrolyte in solution and the subsequently added anionic polyelectrolyte. These complexes can probably act as a bonding agent between the fibres (Buchhammer et al. 1994, Kekkonen et al. 2001, Mende et al. 2002, Picart et al. 2002, Chen et al. 2003, Lavalle et al. 2004). Hence, these systems are not pure PEMs but a mixture of PEM and PECs.

3.3.2 Polyelectrolyte complexes

Mixing of solutions of cationic and anionic polyelectrolytes leads to a spontaneous formation of complexes due to the entropically favourable release of the counterions when the charges of the polyelectrolytes are neutralised by association with each other. In addition to the electrostatic interactions between the oppositely charged polyelectrolytes and to the liberation of the counterions also other interactions such as hydrogen bonding and hydrophobic interactions play a role in the polyelectrolyte complex (PEC) formation (Thünemann et al. 2004). Obviously, the PEC particles consist of a charge neutralised core surrounded by a shell of the excess component, which stabilise the particles against further aggregation (Dautzenberg and Jaeger 2002). Numerous factors such as the nature, charge density and molecular weight of the polyelectrolyte (Mende et al. 2002, Hubbe et al. 2005, Salmi et al. 2007), charge ratio between polyelectrolytes, solution properties such as ionic strength and pH, addition order (Dautzenberg and Jaeger 2002, Chen et al. 2003), agitation (Hubbe et al. 2005) and ageing (Buchhammer et al. 1999, Mende et al. 2002, Chen et al. 2003, Hubbe 2005, Hubbe et al. 2005) affect the complexation between anionic and cationic polyelectrolytes and further the adsorption of the complexes and the properties of the adsorbed layer. However, in this chapter the emphasis is on the factors studied in this thesis, i.e., the charge density of the polyelectrolytes, the mixing ratio and ionic strength.

The association between the polyelectrolytes with low charge density is weak and the complexes are highly swollen but under shear they may disperse as colloidal particles. From high-charge polyelectrolytes stable and well-defined, insoluble complexes that precipitate as colloidal particles or macroscopic flocs are formed due to the strong electrostatic interactions (Kramer et al. 1998, Stenius 2000, Mende et al. 2002, Gärlund et al. 2007).

Complex formation is highly dependent on the mixing ratio of the components. Complexes with a molar charge ratio of less than about 0.5 are charged and stable whereas complexes with a molar ratio above this value are rather unstable due to the strong aggregation and macroscopic flocculation especially in the 1:1 stoichimetry (Petzold and Lunkwotz 1995). If the charge ratio is increased again after precipitation the complexes become soluble again (Petzold et al. 1998, Hubbe 2005, Hubbe et al. 2005).

The effect of salt on the complex formation is complicated and depends strongly on the type and charge density of the polyelectrolytes and the mixing ratio (Dautzenberg and Karibyants 1999, Dautzenberg 2000, Dautzenberg and Jaeger 2002). For complexes formed by high-charge polyelectrolytes the addition of salt causes mainly secondary aggregation and flocculation. Increasing the mixing ratio closer to 1:1 stoichiometry promotes stronger aggregation at lower salt concentration. Addition of salt leads to dissolution of the PECs with lower charge density.

PEC formation can be utilised in various applications, such as in microencapsulation (Lee et al. 1997), in membranes (Zhumadilova et al. 2001) and in waste water cleaning (Buchhammer et al. 2000). In the papermaking process the PECs affect the bonding of the fibre network. The complexes have been found to increase the molecular contact area in the fibre-fibre joint which strengthens the joint and further significantly increases the strength properties of paper made from the treated fibres (Gärlund et al. 2003, Gärlund et al. 2005, Torgnysdotter and Wågberg 2006, Vainio et al. 2006). Sheet formation, retention and dewatering have also been found to improve when PECs are used in papermaking applications (Petzold and Lunkwitz 1995, Bessonoff et al. 2006, Xiao et al. 2006).

3.4 Enzyme interactions with cellulose and lignin

Enzymes are proteins that catalyse chemical reactions in living organisms. Enzymes work by lowering the activation energy for reactions, thus increasing the rate of the reaction. As all catalysts, enzymes are not consumed by the reactions. Enzyme activity can be affected by temperature, chemical environment and substrate concentration (Kuby 1991, Pandey et al. 2005).

Enzymes consist of long, linear chains of amino acids that form a three-dimensional, globular structure. Each amino acid sequence produces a specific structure which has unique properties. Enzymes are usually very specific to the catalysis reactions and the substrates that are involved in these reactions (Kuby 1991, Pandey et al. 2005).

The use of enzymes has become very prominent in papermaking because they are highly selective in their action and have a negligible environmental impact. The main application possibilities of enzymes in pulp production is to save energy and to replace harmful chemicals, mill process water recycling and mill effluent treatment. Enzymes are also widely used in deinking technology for increasing the recyclability of secondary fibre and in pulp treatment to improve paper properties and facilitate papermaking through enhanced pitch control (Widsten and Kandelbauer 2008). Wider application of enzymes is restricted by their cost and availability. Pulp bleaching by enzymes can be done by using hemicellulolitic enzymes (Suurnäkki et al. 1994), such as xylanase, to degrade the hemicellulose from the pulp, or by using oxidative enzymes, mainly laccase (Call and Mücke 1997) and peroxidase (Moreira et al. 2003), to delignify pulp fibres. Cellulases (Suurnäkki et al. 2000) and hemicellulases are used in fibre modification, for example to improve beating, to enhance drainage and to improve certain strength properties, and also in deinking to remove the ink from fibres (Elegir et al. 2000, Marques et al. 2003). Enzymatic debarking, enzymatic beating and reduction of vessel picking with enzymes as well as removal of shives, bio-film build-up and slime have also been developed (Bajpai 1999).

Laccases are multicopper enzymes belonging to the group of blue oxidases (Thurston 1994, Call and Mücke 1997). The crystal structure of the *M. albomyces* laccase in Figure 3.3 shows that the enzyme consists of three domains, indicated as red, green and blue.

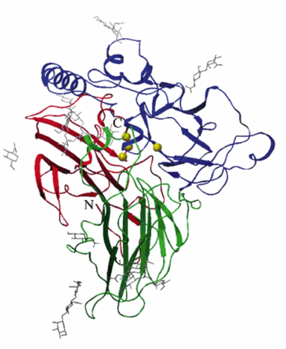


Figure 3.3. *Three-dimensional structure of M. albomyces laccase. Carbohydrates are shown as gray sticks. The red domain includes residues that participate in the binding of coppers at the trinuclear site and the green domain contains residues that take part in substrate binding. The blue domain (C) contains residues that participate in the binding of coppers at the mononuclear and the trinuclear site, as well as in substrate binding. The mononuclear site is located entirely in domain C, and the trinuclear site is located at the interface between the red and blue domains. The diphenolic substrate-binding site is located in the cleft between the green and blue domains. (Hakulinen et al.2002).*

Laccases catalyse oxidation of a wide variety of organic and inorganic compounds, typically different phenols with various substitutes (Gianfreda et al. 1999, Widsten and Kandelbauer 2008). In oxidation the substrate loses a single electron and forms a free radical (Eq. 1). Laccases are capable of catalysing a one-electron oxidation of phenolic hydroxyl groups by reducing O_2 and yielding phenoxy radicals and water (Felby et al. 1997).

$$4\,\mathrm{Phe{-}OH} + \mathrm{O_2} \xrightarrow{\text{laccase}} 4\,\mathrm{Phe{-}O^{\bullet}} + 2\,\mathrm{H_2O} \qquad (1)$$

The formed radicals are assumed to be highly reactive and long-lived and they can possibly be exploited in functionalisation of wood fibres by various means. In lignin-rich pulps, laccases can be used to activate the surface lignin by radicalisation (Felby et al. 1997, Lund et al. 1998, 2003, Grönqvist et al. 2003). As a result of lignin oxidation, phenoxy radicals are formed in the lignin matrix (Felby et al. 1997, Barsberg and Thygesen 1999). However, in lignin activation the laccases could also interact with other wood fibre components and the affinity of the laccases e.g. to cellulose is not yet clear. In order to fully understand the interaction mechanisms of laccases, it is very important to know in detail with which kind of component they interact and under what conditions. The adsorption kinetics and the properties of the laccase layers on lignocellulosic surfaces are valuable concerning the use of enzymes in paper industry.

Formation of phenoxy radicals on the substrate by laccases results in polymerisation, which has been exploited, for example, in fibreboard manufacture (Felby et al. 1997, Kharazipour et al. 1997, Hütterman et al. 2001, Felby et al. 2002, Widsten et al. 2003, Widsten et al. 2004, Widsten and Kandelbauer 2008). The radicalisation of wood fibre surfaces can be utilised in lignin functionalisation when aiming to add desired functionalised groups to fibres for tailoring the wood fibre properties (Chandra and Ragauskas 2001, 2002, Chandra et al. 2004a,b, Grönqvist et al. 2003, 2006, Fackler et al. 2008). Enzymatic functionalisation of fibre surfaces offers, thus, possibilities to create completely new, previously unexploited fibre properties. So far, however, the adsorbed amount of functionalised groups and the structure of the formed have not been studied.

3.5 Lignocellulosic films

Ultrathin model films provide an excellent means to study the chemical and morphological changes taking place in different process conditions. When studying the interactions at a molecular level, pulp fibres can not typically be used, and there is a need for smooth and reproducible model surfaces. Because of its easy availability, smoothness and homogeneity, silica has been widely used as a model substrate when studying the adsorption of polyelectrolytes (Shubin and Linse 1995, Ödberg et al. 1995, Hoogeveen et al. 1996a, Stemme et al. 1999, Wågberg et al. 2004, Samoshina et al. 2005). However, using silica as a model for example for the cellulose fibre surface is not entirely justified. For example the porous structure of the cellulose fibres and the chemistry of cellulose strongly affect the adsorbed amount of polyelectrolytes (Wågberg 2000). In addition, cellulose has a substantially lower charge density than silica.

Model films prepared from the main pulp fibre components, cellulose (Kontturi et al. 2006) and lignin (Norgren et al. 2006, Tammelin et al. 2006), offer an excellent substrate to study the adsorption phenomena in papermaking. The spin-coating technique offers a simple way to prepare model cellulose films, but a drawback of this method is that the spin-coating conditions strongly affect the smoothness of the film (Kontturi et al. 2006). The Langmuir-Blodgett technique (LB) represents a suitable method for preparing thin, smooth and very reproducible cellulose surfaces (Schaub et al. 1993). The advantage of the LB technique compared to spin-coating is the accurate adjustability of film thickness when preparing one monolayer at a time, while still keeping the roughness of the film constant.

Lignin model films have been prepared from sugar cane bagasse (Constantino et al. 1996, Pasquini et al. 2005, Pereira et al. 2007), from Honduran pine lignin (Oliveira et al. 1994), from wheat straw and from milled wild cherry wood (Aguié-Béghin et al. 2002) by using the Langmuir-Blodgett technique and from kraft lignin by using the spin-coating technique (Norgren et al. 2006). The lignin surfaces have also been made from spruce milled wood lignin (MWL, Tammelin et al. 2006). The limited solubility of MWL to all easily evaporating organic solvents restricts the utilisation of the Langmuir-Blodgett technique for preparing model surfaces, so the lignin surfaces from MWL have been prepared by using the spin-coating technique. In spin-coating, the film formation, roughness and thickness can be controlled by varying the solvent properties, solution concentration, acceleration, spinning speed and spinning time (Bornside et al. 1993).

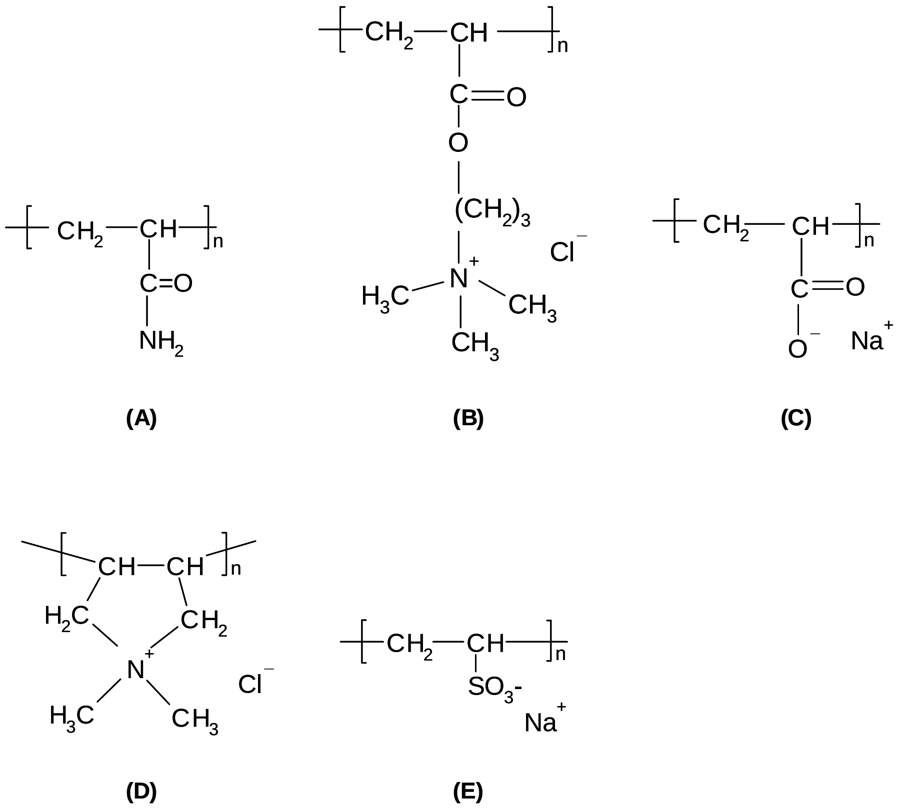
4 EXPERIMENTAL

A general overview of advantages and disadvantages of the materials and methods used in this thesis is given in this chapter. Some theoretical background and the benefits of the methods are also presented. The methods section focuses on the principle and benefits of the main instrument used: the quartz crystal microbalance with dissipation, QCM-D. The modelling of the adsorbed layers is also clarified to some extent. The principle of AFM is also briefly described. The materials and methods used in the experiments are described in more detail in Papers I-V.

4.1 Materials

4.1.1 Polyelectrolytes

The polyelectrolytes used were cationic and anionic polyacrylamides (C-PAM, A-PAM, Kemira Oyj, Finland), cationic polydimethyldiallylammonium chloride (PDADMAC, Allied Colloids Ltd., Yorkshire, England) and anionic sodium polyethylene sulphonate (PESNa, Ciba Specialty Chemicals, Ltd., Bradford, England). The molecular structures of the polyelectrolytes used are presented in Figure 4.1 and the properties of the polyelectrolytes in Table 4.1. A more detailed description of the polyelectrolytes is found in Papers II-III.



(A) (B) (C) (D) (E)

Figure 4.1. *Molecular structure of (A) uncharged PAM, (B) cationic monomer unit of PAM and (C) anionic monomer unit of PAM, (D) PDADMAC and (E) PESNa. C-PAM consists of uncharged and the cationic unit of polyacrylamide, whereas A-PAM consists of uncharged and the anionic unit of polyacrylamide.*

Table 4.1. *Properties of polyelectrolytes.*

Polyelectrolyte	Charge density (meq/g)	Charge density (mol-%)	Molecular weight $\times10^6$ (g/mol)
C-PAM$_{LC-LMW}$[a]	0.9	7	0.2
C-PAM$_{LC-HMW}$[b]	1.0	9	2.4
C-PAM$_{MC-HMW}$[c]	1.8	17	2.4
C-PAM$_{MC-LMW}$[d]	2.8	33	0.3
PDADMAC$_{HC-LMW}$[e]	6.1	100	0.4
A-PAM$_{MC-HMW}$[c]	1.8	13	2.5
PESNa$_{HC-LMW}$[e]	7.7	100	0.02

a low charge density and low molecular weight
b low charge density and high molecular weight
c medium charge density and high molecular weight
d medium charge density and low molecular weight
e high charge density and low molecular weight

4.1.2 Enzymes

Two different purified enzymes, *Trametes hirsuta* laccase (ThL) and *Melanocarpus albomyces* laccase (MaL), were used in the enzyme experiments. *T. hirsuta* laccase was produced according to Poppius-Levlin et al. (1999) on a glucose-yeast extract medium using 1-hydroxybenzotriazole as inducer. *T. hirsuta* laccase was purified by anion exchange chromatography at pH 4.8 using sodium acetate as a buffer and characterised according to Rittstieg et al. (2002). *M. albomyces* laccase was cultivated in flasks at 37 ºC on a rotary shaker (160 rpm) according to Kiiskinen et al. (2002). Extracellular laccase activity was assayed daily from the culture filtrate and the enzyme was collected when laccase activity reached its maximum. The purification of *M. albomyces* laccase was made according to Kiiskinen et al. (2002). Enzyme activities were determined using 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) as a substrate (Niku-Paavola et al. 1988). The protein concentration was determined by amino acid analysis for the *M. albomyces* laccase (Aminosyraanalyscentralen, Uppsala University, Sweden) and by using the Bio-Rad DC protein assay kit (Bio-Rad, Richmond, USA) with bovine serum albumin as a standard for the *T. hirsuta* laccase. The purification and characterisation of laccases are described in more detail in Papers IV and V. The adsorption of ferulic acid (4-hydroxy-3-methoxycinnamic acid, $C_{10}H_{10}O_4$, Fluka Chemika) (Figure 4.2) on laccase-treated lignin surface was studied in Paper V.

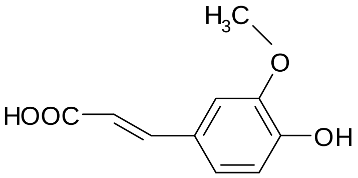


Figure 4.2. *Molecular structure of ferulic acid.*

4.1.3 Model surfaces

The QCM-D crystals were AT-cut quartz crystals supplied by Q-Sense AB, Västra Frölunda, Sweden. The thickness of the crystals was 0.3 mm, the fundamental frequency (f_0) was 5 MHz and the sensitivity constant (C) was 0.177 mg/m^{2}Hz. The silica surfaces were prepared by vapour deposition and the polystyrene surfaces by spin-coating by the supplier.

Trimethylsilyl cellulose (TMSC) was prepared by silylation of microcrystalline cellulose powder from spruce (Fluka Chemika) with hexamethyl disilazane (Cooper et al. 1981, Greber and Paschinger 1981). The preparation and characterisation of TMSC with Fourier transform infrared spectroscopy (FTIR) and with electron spectroscopy for chemical analysis (ESCA) are described in Paper I.

The cellulose surfaces were prepared by depositing trimethylsilyl cellulose onto a hydrophobic polystyrene-coated gold crystal by using the horizontal Langmuir-Schaefer (LS) dipping technique as described in Paper I (Figure 4.3). TMSC was dissolved in chloroform and spread with a syringe on the water surface in a Langmuir trough. After chloroform evaporation the water surface contains only TMSC molecules. The monolayer of TMSC was compressed with the barriers (Figure 4.3a) so that it formed a continuous film. The surface pressure and surface area were monitored simultaneously.

The QCM-D crystal, mounted on the dipper with a suction pad, was horizontally pressed onto the TMSC film (Figure 4.3b). The crystal was kept in contact with the TMSC monolayer for 30 s. Then the crystal was lifted (Figure 4.3c) and allowed to dry in air for 15 min, continuing again the contact with the TMSC layer. Throughout the deposition cycle the barriers kept the surface pressure in the trough at a constant value of 15 mN/m. Theoretically, during one dipping cycle a bilayer of TMSC is attached on the polystyrene surface, as shown in Figure 4.3 d-e. Because of the stiff structure of the TMSC molecules one dipping cycle does not lead to a fully covering bilayer film, but a network of TMSC molecules is attached on the surface. This network contains holes which are covered when the number of dipping cycles is high enough, meaning 30 cellulose layers. The deposited TMSC on the polystyrene crystal was converted into cellulose by desilylation the crystal for 3 minutes in the atmosphere of an aqueous 10% hydrochloric acid solution (Schaub et al. 1993). Thereafter, the cellulose-coated crystals were allowed to swell in the appropriate electrolyte solution for at least 12 hours before experiments.

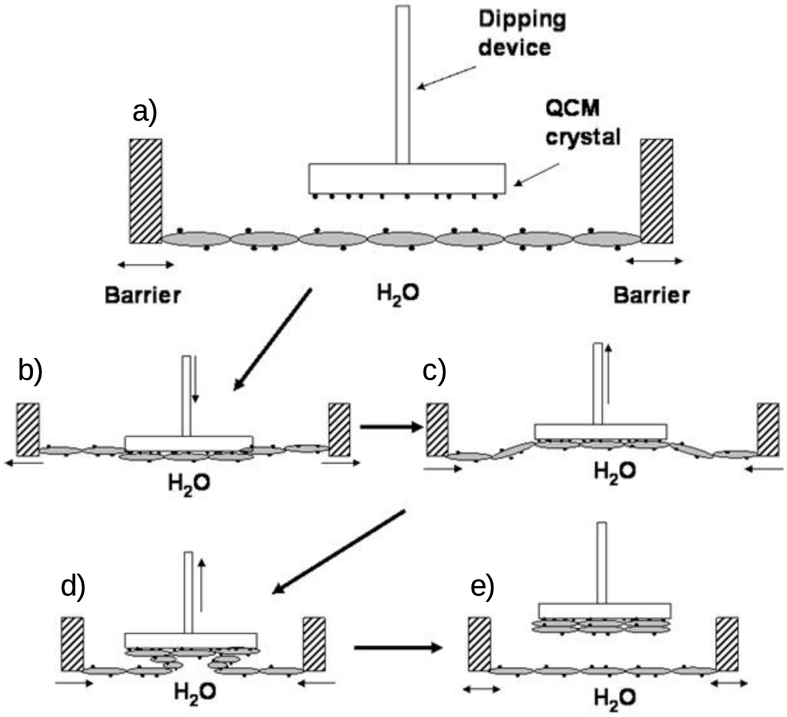


Figure 4.3. *Schematic drawing of the horizontal Langmuir-Schaefer dipping procedure (Paper I). Horizontal arrows represent the barrier movements and vertical arrows the movements of the dipping device during one dipping cycle. Grey ovals illustrate the TMSC molecules and black spots the hydrophobic parts of TMSC and polystyrene. The molecules are not drawn to scale.*

Milled wood lignin (MWL, KCL Science and Consulting, Espoo, Finland) was isolated from Norway spruce (*Picea abies*) by using a slight modification of the Björkman method (Björkman 1956), including an ultrasonic extraction step at 15 ºC. The lignin surfaces examined in Papers IV and V were prepared by spin-coating 0.5 w-% spruce MWL solution dissolved in 1,4-dioxane on the QCM-D polystyrene crystal (Tammelin et al. 2006). The lignin-coated crystals were allowed to swell and stabilise in the appropriate electrolyte solution at least 12 hours before experiments.

4.2 Methods

4.2.1 Quartz crystal microbalance with dissipation

The adsorption of polyelectrolytes and enzymes was studied with a quartz crystal microbalance with dissipation (QCM-D), using the chambers D300 and E4 (Q-Sense AB, Västra Frölunda, Sweden). The basic principle of this instrument has been described by Rodahl et al. (1995) and Höök et al. (1998). QCM-D allows simultaneous measurement of the adsorbed amount and viscoelastic properties of the adsorbed layer. The crystal oscillates at a resonance frequency, f_0, without adsorbate. When material adsorbs on the surface of the crystal, the frequency is lowered to f. If the adsorbed mass is evenly distributed, rigid and small compared to the mass of the crystal, $\Delta f = f - f_o$ is related to the adsorbed mass by the Sauerbrey equation (Sauerbrey 1959).

$$\Delta m_{Sauerbrey} = -\frac{C\Delta f}{n} \qquad (2)$$

where Δm is the adsorbed mass per unit surface, n is the number of the overtone used in the measurement and C is a sensitivity constant (0.177 mg/m^{2}Hz).

The thickness of the adsorbed layer, $d_{Sauerbrey}$, can be calculated by combining the estimated mass of the adsorbed layer with the assumed density of the adsorbed film (ρ).

$$d_{Sauerbrey} = \frac{\Delta m_{Sauerbrey}}{\rho} \qquad (3)$$

When the source driving the oscillation of the crystal is cut off, the amplitude of the oscillation decays due to dissipation of energy in the crystal, the adsorbed layer and the surrounding solution. The decay rate depends on the viscoelastic properties of these materials. Dissipation is characterised by the dissipation factor D, which is defined by

$$D = \frac{E_{diss}}{2\pi E_{stor}} \qquad (4)$$

where E_{diss} is the dissipated energy during one oscillation cycle and E_{stor} is the total energy stored in the oscillation. With the QCM-D the change in dissipation, $\Delta D = D - D_0$ is measured, where D_0 is the dissipation factor of the pure crystal in solvent before adsorption and D is the dissipation factor after adsorption. In this way the relative stiffness or conformation of the adsorbed layer can be determined. High dissipation values reflect a thick and loose adsorbed layer, while a thin and rigid layer vibrates with the crystal, indicating a low dissipation factor.

The Sauerbrey equation (Eq. 2) takes into account only the closely adhered layer which moves with the crystal (Stengel et al. 2005). For non-rigid adsorbed layers the Sauerbrey relation is not valid. High dissipation values mean that the soft film cannot function as a fully coupled oscillator. The outer layers of the film, far away from the surface do not follow the oscillation of the crystal. Thus, the Sauerbrey relation between Δf and Δm underestimates the thickness of the film: the higher the dissipation, the greater the underestimation. By measuring the Δf and ΔD at several overtones it is possible to estimate the film thickness by modelling. The viscoelastic layers can be modelled using a Maxwell or a Voigt description. These models are very simple and the viscoelastic behaviour is simulated in these models, using elastic spring and viscous dashpots in parallel (Voigt) or in series (Maxwell).

The interpretation of the viscoelastic properties of the adsorbed layer (Figure 4.4) in this work is based on the Voigt model presented by Voinova et al. (1999). Because of the simplicity of the model several assumptions have to be made:

i. the quartz crystal is purely elastic
ii. the surrounding solution is purely viscous and Newtonian
iii. the adsorbed film has uniform thickness and density
iv. there is no slip between the adsorbed layer and the quartz crystal
v. viscoelastic properties are independent of frequency

In the modelling, the known parameters are the fluid density and viscosity and the guessed parameter is the density of the adsorbed layer. Using the known parameters of the fluid, the assumed layer density as well as the measured changes in frequency and in dissipation, it is possible to estimate the viscosity, shear modulus and thickness of the adsorbed layer (Figure 4.4).

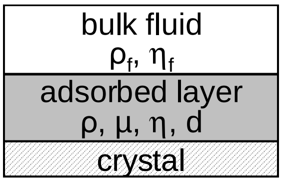


Figure 4.4. *Schematic drawing of the viscoelastic adsorbed layer on the quartz crystal. The viscoelastic layer is determined by the density* (ρ)*, viscosity* (η)*, shear modulus* (μ) *and thickness (d) and the bulk liquid above the adsorbed layer by the density* (ρ_f) *and viscosity* (η_f) *of the fluid.*

The conformational changes during the adsorption process can be considered by plotting the change in dissipation as a function of change in frequency, where time as a variable is eliminated. The steepness of the slope of the *D-f* plot describes the softening or packing of the layer structure during the adsorption. High $\Delta D/\Delta f$ values describe loose layers, while thin and flat layers have low $\Delta D/\Delta f$. A curved and irregular graph can be interpreted as conformational changes during the adsorption.

The QCM-D results were fitted to the Voigt model at several overtones (3rd, 5th, 7th, 9th, 11th and 13th), using the Q-Tools program (Q-Sense AB, Västra Frölunda, Sweden). The accuracy of the estimation improves and the noise-to-signal ratio decreases when several overtones are used in the modelling. The best fit for the Voigt model was obtained by using a constant fluid density (1.05 g/cm^3), viscosity (1.3×10^{-3} Ns/m^2) and density of the adsorbed polyelectrolyte layer (1.15 g/cm^3, Notley et al. 2005, Dutta and Belfort 2007). These assumptions are not all correct, since the properties of the polyelectrolyte solutions used in this thesis were not constant. Despite using only one constant value for the density and viscosity of the polyelectrolyte solution and further for the density of the adsorbed layer and that the density of the adsorbed layer changes as a function of the distance from the surface, comparisons between different systems could be made.

The Sauerbrey equation (Eq. 2) calculates the adsorbed mass with the coupled water inside the adsorbed layer. Therefore, the adsorbed mass detected by QCM-D is usually higher compared to the adsorbed mass detected with optical techniques, e.g. stagnation point adsorption reflectometry (SPAR, Wågberg and Nygren 1999, Geffroy et al. 2000, Wågberg et al. 2004,) XPS (Rojas et al. 2000) and ellipsometry (Shubin and Linse 1995, Ödberg et al. 1995, Stemme et al. 1999, Samoshina et al. 2003). When comparing the results obtained with these different methods it is important to remember that they measure different phenomena, which consequently will affect the results. The optical techniques are not sensitive to water associated with the adsorbed polymer layer. If the mass of the trapped solvent could be determined in QCM measurements, it would enable determination of the amount of solvent attached to the adsorbed layer as well as the mass of the adsorbed film alone. One way to do this is to utilise the density difference between a deuterated and a non-deuterated solvent (Höök et al. 2001, Graig and Plunkett 2003). The other way is to compare results obtained from different methods (Höök et al. 2001, 2002, Macakova et al. 2007, Naderi et al. 2008).

4.2.2 Atomic force microscopy

Atomic force microscopy (AFM, Binning et al. 1986) was used to study the morphology of cellulose and lignin substrates after enzymatic modification (Paper IV) and to obtain information on the changes in surface properties after the addition of ferulic acid on the laccase-treated lignin (Paper V).

The AFM consists of a micro-scale cantilever with a sharp tip at its end that is used to scan the sample surfaces. The AFM can be operated in a number of modes, depending on the application. The imaging modes can be divided into static (contact) mode (Martin et al. 1987, Zhong et al. 1993) and a variety of dynamic, non-contact modes (Albrecht et al. 1991, García and Pérez 2002). In static mode the force between the tip and the surface is kept constant during scanning by maintaining a constant deflection, whereas in the dynamic mode the tip is oscillated at its resonance frequency in proximity to the surface. In the tapping mode the frequency of the oscillation is kept constant with a piezoelectric driver and the changes in amplitude are monitored. The tip is in contact with the sample only briefly. The forces affecting between the tip and surface cause a reduction in the amplitude. The amplitude is kept constant by moving the sample with the piezo. A topography image is generated by detecting the movement of piezo during the scanning.

The oscillation of the cantilever as well as the oscillation of the piezo, which is used to vibrate the tip, is assumed to be harmonic. The phase angle between the tip and the piezo oscillation is initially set to 90º. The amplitude decreases and the phase angle of the harmonic waves changes when the tip approaches the sample because of the interactions between the tip and surface. By recording the phase shift during scanning a phase image is obtained simultaneously with the topography image. The phase image offers information about the relative differences in elasticity and adhesive properties of the sample surface. (Bar et al. 1997, Spatz et al. 1997).

AFM measurements were made with a Nanoscope IIIa Multimode scanning probe microscope (Digital Instruments Inc., Santa Barbara, CA, USA). The images were scanned in tapping mode in air using silicon cantilevers (Pointprobes, type=NCH, delivered by Nanosensors, Neuchald, Switzerland). No image processing except flattening was done and at least three areas on each sample were measured. The root mean square (rms) roughness of the laccase-treated substrates was determined from the 1 µm^2 AFM topography images.

4.2.3 Other methods

The dynamic viscosity of the polyelectrolyte solutions was determined by using a computer-controlled Ubbelohde capillary viscometer (Schott-Geräte, AVS 350). The viscosities were analysed in 1 M NaCl at 25 ºC, and at least two parallel measurements of each sample were made. The charge density of the polyelectrolyte solutions was determined according to the procedure applied by Koljonen et al. (2004). Electrophoretic mobilities of PECs were analysed using a Coulter Delsa 440 instrument (Doppler Electrophoretic Light-scatter analyser).

5 RESULTS AND DISCUSSION

The most important findings of this work are summarised in this chapter. First, the lignocellulosic model films are characterised. Second, the effect of ageing of polyacrylamide solutions is studied since the properties of some polyelectrolyte solutions have been found to change with time and it is very important to standardize the conditions used. Third, the adsorption of polyelectrolytes, polyelectrolyte multilayers (PEMs) and complexes (PECs) are considered. Finally, the modification of lignocellulosic surfaces by laccases and the adsorption of ferulic acid on laccase-activated lignin are summarised. More detailed results can be found in Papers I-V.

5.1 Characterisation of lignocellulosic model films

The adsorption experiments with single polyelectrolytes, PEMs and PECs were made on the silica and Langmuir-Schaefer cellulose surfaces in order to find out the properties and structure of the adsorbed layers on a smooth reference surface (silica) as well as on a cellulose surface. The laccases were adsorbed on cellulose and spin-coated lignin films since the laccases have been known to activate the surface lignin by radicalisation. The laccases can also interact with other wood fibre components and the affinity of the laccases e.g. to cellulose is not yet clear.

5.1.1 Langmuir-Schaefer cellulose film

The morphology, coverage, chemical composition and wetting of the cellulose model film prepared by the horizontal Langmuir-Schaefer (LS) dipping method were characterised using AFM, ESCA, and contact angle measurements (results shown in Paper I). A smooth and thin cellulose surface was achieved with an rms roughness of approximately 0.4 nm and with a film thickness of about 12 nm estimated from AFM images.

The swelling of LS-coated cellulose film increases upon increasing sequentially the ionic strength (Figure 5.1). The QCM-D instrument shows the water penetration into the cellulose film and swelling as a decrease in frequency and an increase in dissipation. After 16 hours' water penetration into the cellulose, the structure was stabilised, whereas the changes in dissipation ($\Delta D<0.5\times10^{-6}$) were very small. This indicates slight swelling of the film but no major layer softening. At this stage the amount of water inside the film is 2.2 mg/m^2, calculated using the Sauerbrey equation (Eq. 2).When the electrolyte concentration was slightly increased (1 and 10 mM NaCl), the ΔD of the cellulose film decreased. This suggests some deswelling although the Δf still decreases.

At higher electrolyte concentrations (100 - 1000 mM NaCl) the properties of the electrolyte solution screen the effects of water penetration and swelling/deswelling of the film. The very sharp changes in frequency and dissipation observed immediately after electrolyte additions probably result from the changes in density and viscosity of the liquid rather than the water penetration and swelling/deswelling of the film.

The proper use of the Langmuir-Schaefer cellulose surface requires taking into account the slow stabilisation and swelling of the cellulose film. Equilibrating the film in an appropriate electrolyte solution is a requirement for the reproducible adsorption studies. After overnight stabilisation of the film in the appropriate electrolyte solution a very stable cellulose surface was obtained that was used in the adsorption studies of polyelectrolytes and enzymes.

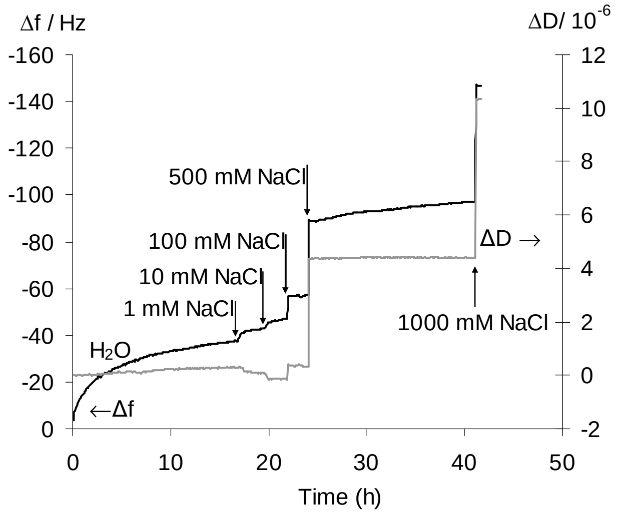


Figure 5.1. *Change in frequency and in dissipation as a function of time during the swelling of cellulose film in water/aqueous electrolyte solutions with sequentially increasing ionic strength at pH~8 (Paper 1).* f_0*=5 MHz, n=3.*

5.1.2 Lignin film

Spin-coated lignin surfaces were prepared according to the procedure described by Tammelin et al. (2006). The spin-coated lignin films were prepared for QCM-D measurements and therefore the stability and repeatability were studied by using the QCM-D instrument. The surfaces were also analysed by AFM and ESCA (Tammelin et al. 2006).

The AFM images showed that after four spin-coating layers the lignin film was fully covered and moderately smooth (Tammelin et al. 2006). The rms roughness was approximately 0.4 nm and the film thickness between 200 and 300 nm. The surface chemical analysis made by

ESCA showed that atomic compositions of spin-coated films were well in accordance with theoretical values (Gray 1978). The swelling and stability measurements by QCM-D showed that some water and electrolyte solution (100 mM NaCl) penetrated into the lignin structure, which could be seen as a decrease in the change in frequency, but the dissipation value did not change during the experiments, indicating that no layer softening occurred (Tammelin et al. 2006). The lignin films were fully stabilised after six hours. When equilibrated in pure water the amount of water inside the lignin film was 1.4 mg/m^2 and in 100 mM NaCl the amount of electrolyte solution inside the film was 0.5 mg/m^2, calculated using the Sauerbrey equation (Eq. 2). Based on these experiments the lignin films seemed to be very stable in the conditions used in this thesis if the films were allowed to swell in the appropriate solutions overnight. The films were also very repeatable according to the adsorption experiments with hemicellulose (Tammelin et al. 2006).

5.2 Ageing of polyelectrolyte solution

The properties of some polyelectrolyte solutions change with time. The ageing of polyacrylamide solutions has been found to occur over a period of days because of the disentanglement of the polymer molecules as a function of time (Narkis and Rebhun 1966, Shyluk and Stow 1969, Gray et al. 2007). The polymer molecules can become entangled during dissolution of polymer powder, achieving an increase in solution viscosity. During ageing the polymer chains start to disentangle due to the polymer motion which decreases the solution viscosity. The degree of entanglement is higher the higher the molecular weight of the polymer or the concentration of the dissolved polymer is. Precipitation and re-solubilisation studies of the aged polymer solution have been indicated that the drop in viscosity is reversible and therefore not a result of degradation of the polymer chains (Narkis and Rebhun 1966). However, the ageing does not affect the flocculation performance and floc strength which indicates that no significant degradation of the polyelectrolyte chains occurs with time (Gray et al. 2007).

The properties of the polyacrylamide solutions used in this thesis also changed as a function of time. The working solutions (0.2 g/l) were diluted from the 5 g/l stock solution. The viscosity changes of the stock solution after 10 h up to 6 days ageing have been found to be minimal (Owen et al. 2002). Therefore in this thesis, the stock solutions were allowed to stabilise overnight before further dilution and the maximum age for the stock solution was not more than one week. The working solution was allowed to age and the dynamic viscosity and charge density of the 0.2 g/l solution in 1 mM NaCl as well as the adsorption on a silica surface by QCM-D were measured (unpublished data).

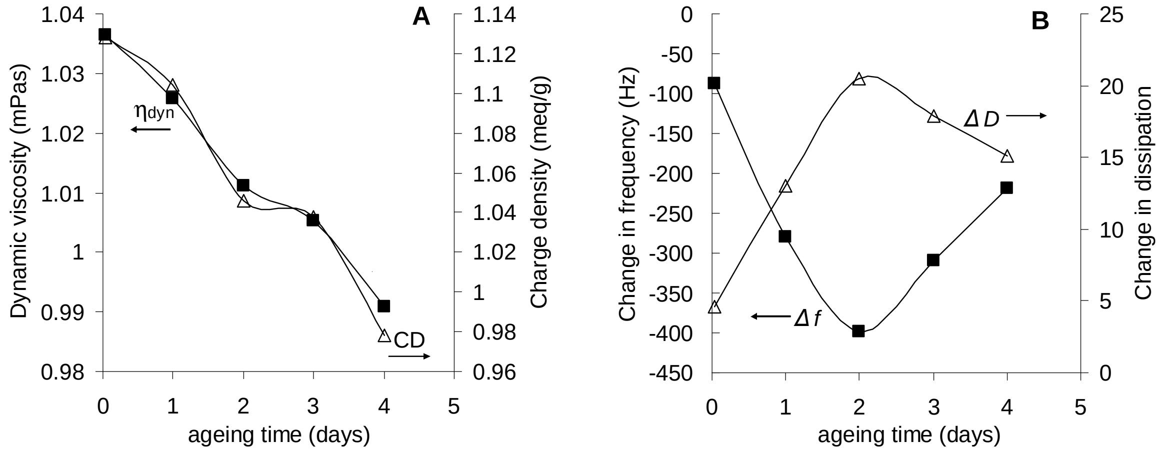


Figure 5.2. *A) Dynamic viscosity and charge density for solution of 0.2 g/l C-PAM$_{LC\text{-}LMW}$ and B) change in frequency and in dissipation for adsorption of 0.2 g/l C-PAM$_{LC\text{-}LMW}$ in 1 mM NaCl on silica as a function of the age of the C-PAM solution (unpublished data). The adsorption time in the QCM-D experiments was 20 min. f_0=5 MHz, n=3.*

The charge density and dynamic viscosity of the polyacrylamide solution decreased almost linearly with the age of the solution (Figure 5.2). The decrease in charge density indicates that the cationically charged monomer unit was not stable in prevailing conditions (1 mM NaCl). On the basis of the literature (Narkis and Rebhun 1966, Shyluk and Stow 1969, Gardner et al. 1978, Hecker et al. 1998, Owen et al. 2002, Gray et al. 2007) a significant decrease in solution viscosity is probably result from the disentanglement of the polyelectrolyte chains. A clear maximum was seen in the adsorption curves of QCM-D results as a function of the ageing time of the polyacrylamide solution. First, the final Δf decreased and ΔD increased until after two days the Δf started to increase and ΔD to decrease. There is no clear explanation for this behaviour. However, it is evident that the degree of entanglement and polyelectrolyte conformation are varying and hence, influence the adsorbed amount as a function of the ageing time of the polyelectrolyte solution. A conclusion based on these measurements was that the age of the C-PAM solution for the adsorption measurements must be the same in all experiments. Therefore, all polymer dilutions were made from the stock solution exactly one hour before use.

5.3 Adsorption of polyelectrolytes

By using QCM-D the adsorption phenomenon of single cationic polyelectrolytes, the factors affecting the adsorption and the conformation and viscoelastic properties of the adsorbed polyelectrolyte layers are considered in this chapter. More detailed results can be found in Paper II.

5.3.1 Effect of polyelectrolyte and surface properties on adsorption

To determine the optimum polyelectrolyte concentrations to be used in the subsequent adsorption measurements, the change in frequency as a function of added polyelectrolyte was measured (Paper II). The changes in frequency of the polyelectrolytes with low charge density at low ionic strength on the SiO_2 surface were high (diamonds and squares in Figure 5.3). As expected, during the adsorption of high-charge polyelectrolytes on silica (spheres and triangles in Figure 5.3) a plateau is reached already after the first addition and the total Δf was very low. Polyelectrolyte with higher charge density has a more extended conformation in solution and it adsorbs in a flatter conformation. As a consequence less polyelectrolyte is needed for surface charge neutralisation (Evers et al. 1986, van de Steeg et al. 1992, Shubin and Linse 1995, Rojas et al. 2002).

The adsorption of low-charge C-PAMs was clearly lower on cellulose than on SiO_2 (Figure 5.3, right). Because of the difference in charge density, less polyelectrolyte was required to neutralise the cellulose surface than the SiO_2 surface. On the other hand, the change in frequency upon adsorption of medium- and high-charge polyelectrolytes on cellulose was slightly higher than that on silica. Theoretically, an increase in the surface charge density leads to higher adsorption of polyelectrolyte because the polyelectrolyte adsorbs until it compensates for the surface charge (van de Steeg et al. 1992, Böhmer et al. 1990). This was also the case for the low-charge polyelectrolyte whereas the differences in conformation, in the amount of coupled water and in surface porosity seemed to dominate the Δf for high-charge polyelectrolyte.

The adsorbed amounts at the end of the isotherms in Figure 5.3, calculated from Eq. 2, were approximately 8.3 mg/m^2 for low-charge C-PAMs and between 0.4-1.4 mg/m^2 for medium- and high-charge polyelectrolytes on SiO_2. On cellulose the corresponding adsorbed amounts varied between 2.7-5.7 mg/m^2 for all polyelectrolytes. Compared to the adsorbed amounts obtained using ellipsometry (Shubin and Linse 1995, Ödberg et al. 1995), XPS (Rojas et al. 2000) or stagnation point adsorption reflectometry (Wågberg and Nygren 1999, Geffroy et al. 2000, Wågberg et al. 2004), higher adsorbed amounts were obtained in this study (Paper II). The main reason for this difference is the coupled water included in the mass calculated with QCM-D, although slight differences in the molecular weight and charge density of the polyelectrolytes used obviously also affect the results.

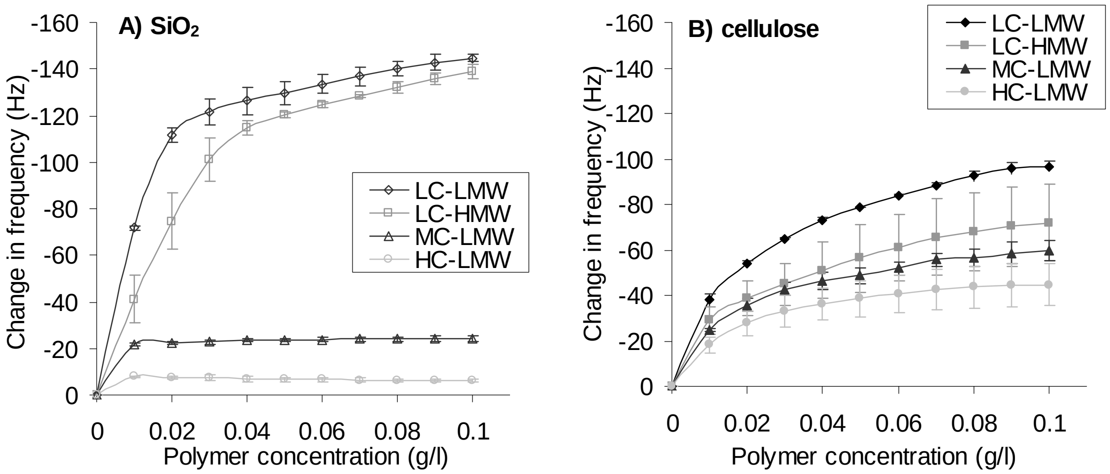


Figure 5.3. *Adsorption isotherms of polyelectrolytes in 1 mM NaCl on A) SiO_2 and B) cellulose surfaces (Paper II). LC-LMW=C-PAM with low charge density and low molecular weight, LC-HMW=C-PAM with low charge density and high molecular weight, MC-LMW= C-PAM with medium charge density and low molecular weight, HC-LMW=PDADMAC with high charge density and low molecular weight. The additions have been made at 20-minute intervals. f_0=5 MHz, n=3.*

5.3.2 Properties of adsorbed polyelectrolyte layers

The properties of adsorbed polyelectrolyte layers of low- and high-charge polyelectrolytes were examined by plotting the change in dissipation as a function of the change in frequency (Figure 5.4, Paper II). The high-charge polyelectrolyte immediately covers the high-charge silica surface at low ionic strength, which can be seen as a cluster in measurement points, indicating a very flat conformation (Figure 5.4, left). The adsorption of the low-charge C-PAM on silica has two distinct regions. Initially, the dissipation increases slowly with frequency. As the adsorption proceeds, the dissipation increases, while the frequency stays more or less constant and the curve is almost vertical. This type of behaviour has previously been seen for adsorption of a low-charge polyelectrolyte on gold (Plunkett et al. 2002). Probably the last polyelectrolytes reaching the surface adsorb in a more extended conformation compared to the first molecules. The effect of the molecular weight of polyelectrolyte on the adsorption is discussed more in Paper II.

The adsorption behaviour on cellulose is different to the behaviour on SiO_2 (Figure 5.4, left). Two distinct regions for the low- and high-charge polyelectrolytes were found at low salt concentration on cellulose. In the first region, a steep increase in dissipation suggests a viscous layer. When the adsorption proceeds, the curves level off, indicating that the chains collapse, more polymer adsorbs and the layers become more rigid. Probably the first polyelectrolytes that reach the surface attach to the cellulose substrate with only at a few anchoring points giving rise to a very viscous layer (Wågberg et al. 1988).

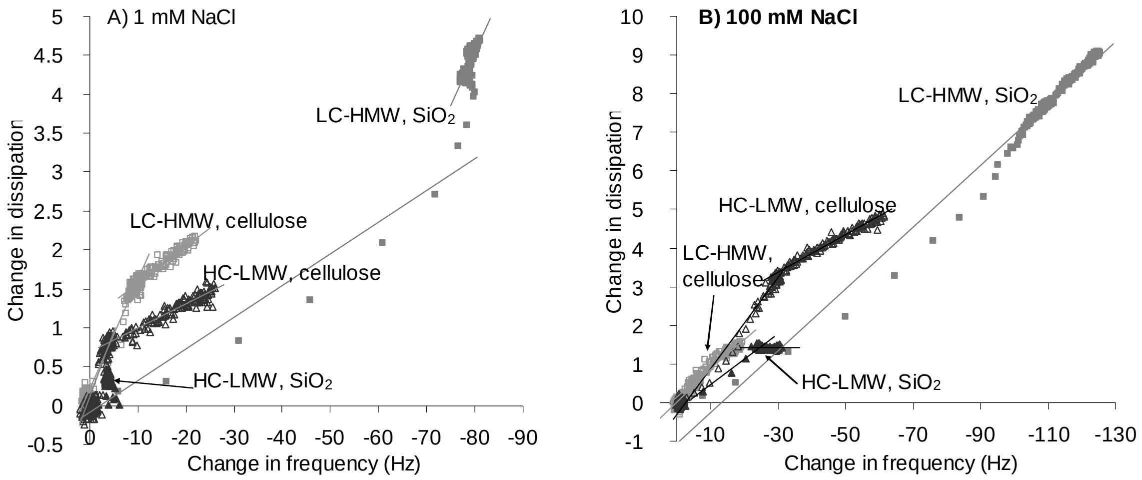


Figure 5.4. *Change in dissipation versus change in frequency for adsorption of 0.5 g/l polyelectrolytes on* SiO_2 *and cellulose in A) 1 mM NaCl and B) 100 mM NaCl (Paper II). The polyelectrolytes used were low-charge and high molecular weight* $C\text{-}PAM_{LC\text{-}HMW}$ *and high-charge and low molecular weight* $PDADMAC_{HC\text{-}LMW}$*.* $f_0=5$ *MHz, n=3, t=60 min.*

At high salt concentration the low-charge polyelectrolyte ($C\text{-}PAM_{LC\text{-}HMW}$) adsorbs with a roughly linear relationship between ΔD and Δf on silica and on cellulose, suggesting no conformational changes during the adsorption process (Figure 5.4, right). High-charge PDADMAC still formed a rather rigid layer on SiO_2, but the conformation changes during adsorption. First, there is a more or less linear relationship between ΔD and Δf. In the second region, the slope is almost horizontal and only Δf changes slightly, suggesting a more rigid polyelectrolyte layer. At a high electrolyte concentration the high-charge polyelectrolyte adsorbs to a greater extent than at a low ionic strength on cellulose. Both the final ΔD and Δf values are higher than at a low salt concentration, but the shapes of the curves are roughly similar.

The rheological properties of low-, medium- and high-charge polyelectrolyte layers were studied by modelling the data with the Q-Tools program (Paper II). By measuring the Δf and

ΔD at several overtones it is possible to estimate the film properties using the Voigt model (Voinova et al. 1999). The Voigt estimation could only be used when the adsorbed layer was thick enough ($D \geq 2\times10^{-6}$). An example of the viscoelastic properties of differently charged polyelectrolytes is given in Figure 5.5. Both viscosity and shear modulus were higher for high-charge PDADMAC in 100 mM NaCl on SiO_2, indicating a more rigid layer (Figure 5.5). Lower-charged C-PAMs formed less rigid and looser layers. The viscosity and shear modulus of the C-PAMs did not change with time, whereas the rheological properties of PDADMAC slightly increased when the adsorption proceeded. These results were in agreement with the observations found in the *D/f*-plot (Figure 5.4).

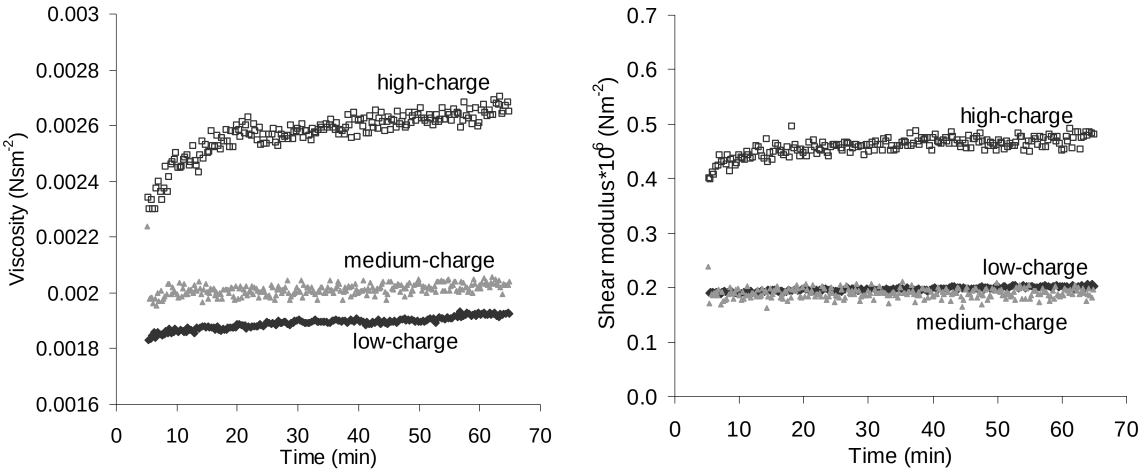


Figure 5.5. *Viscosity and shear modulus as a function of time for adsorption of C-PAM$_{LC-LMW}$, C-PAM$_{MC-LMW}$ and PDADMAC$_{HC-LMW}$ in 100 mM NaCl on SiO_2 determined using the Voigt model (Paper II).*

The thickness of the adsorbed polyelectrolyte layers in Figure 5.6 was estimated using both the equation 3 and the Voigt model (indicated by smaller symbols in the figure), depending on the *ΔD* values (Paper II). As expected, the estimated thicknesses, using the Voigt model were higher than the Sauerbrey thicknesses, especially for dissipative layers.

The estimated thickness of the adsorbed polyelectrolyte layers increased with an increase in ionic strength from 1 to 100 mM NaCl on silica (Figure 5.6). This is as expected, since the polyelectrolytes have a more coiled conformation in solution at higher ionic strength and, consequently, they adsorb in a looser conformation on the surface (Böhmer et al. 1990, Fleer et al. 1993). At very high ionic strength (1000 mM NaCl) the interactions between the polyelectrolyte and the surface become weaker and the adsorption of polyelectrolytes and the thickness of the adsorbed polyelectrolyte layers decreased close to zero (Figure 5.6, Paper II). A similar behaviour has been observed for the adsorption of PDADMAC on cellulosic fibres by Horvath et al. (2006). The layer thicknesses of low-charge C-PAMs were clearly higher than those of the high-charge PDADMAC, which is also well in accordance with theory (Fleer et al. 1993).

The differences between the low- and high-charge polyelectrolyte layers adsorbed on cellulose were not as pronounced as on silica. On cellulose the layer thicknesses of low-charge polyelectrolyte were lower than on silica in contrast to the layer thicknesses of high-charge polyelectrolytes. These results were in agreement with the adsorption isotherms in Figure 5.3.

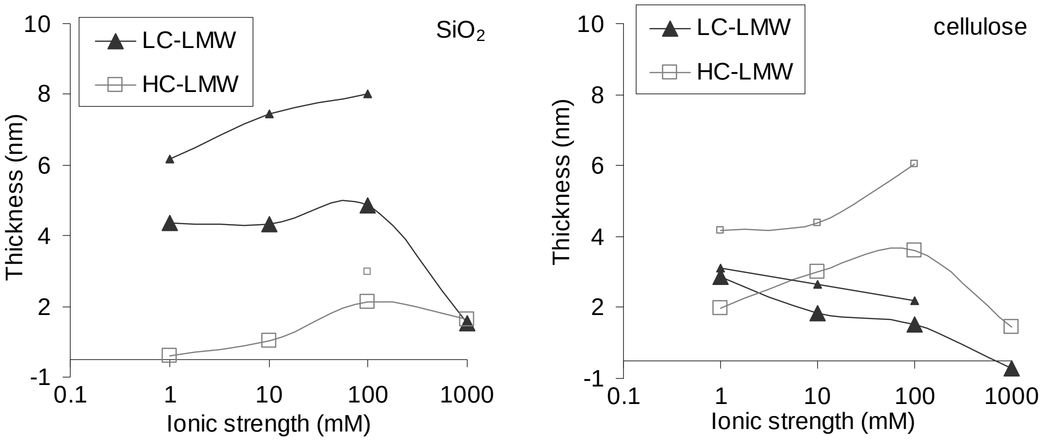


Figure 5.6. *Thickness of adsorbed polyelectrolyte layers as a function of ionic strength on SiO_2 (left) and cellulose (right) substrates determined using the equation 3, (large symbols) and Voigt model (small symbols) (Paper II).*

5.4 Adsorption of multicomponent systems

The most relevant observations of polyelectrolyte multicomponent systems are described in this chapter. PEMs were made by adding layer-by-layer the oppositely charged polyelectrolytes, starting with the cationic polyelectrolyte on SiO_2 and cellulose (Paper III). The PECs were prepared as isotherms, adding the premixed aqueous solutions of oppositely charged polyelectrolytes in various stoichiometric ratios (Paper III).

5.4.1 Polyelectrolyte multilayers

Multilayer formation by medium-charge C-PAM and A-PAM on SiO_2 and on cellulose at two electrolyte concentrations is shown in Figure 5.7 and in Paper III. Both polyacrylamides rather evenly increased the Δf and ΔD on both substrates and at both ionic strengths at the beginning of the multilayer formation. After about four layers a clear difference between adsorption on silica in 1 mM and 100 mM NaCl is observed: at low salt concentration the change in frequency increases with an increase in polyelectrolyte concentration, whereas at high ionic strength the Δf levels off. The ΔD curves, on the other hand, are similar at both salt concentrations. On cellulose the multilayer formation at both electrolyte concentrations resembled the behaviour on silica at low polyelectrolyte concentrations. At high salt and polyelectrolyte concentrations A-PAM mainly increased Δf, while C-PAM increased ΔD.

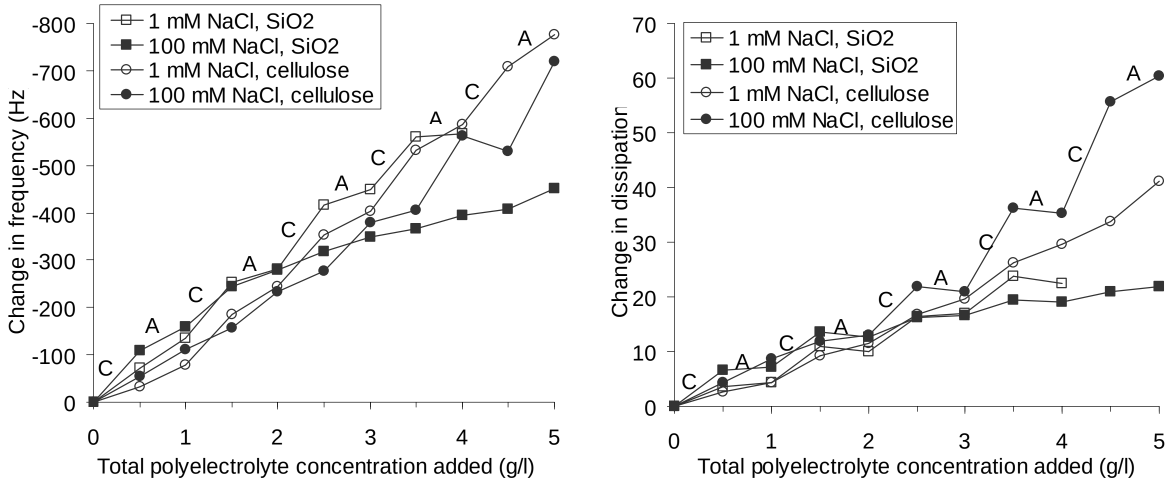


Figure 5.7. *Multilayer formation through sequential additions of 0.5 g/l C-PAM$_{MC\text{-}HMW}$ and 0.5 g/l A-PAM$_{MC\text{-}HMW}$ added at 20-minute intervals, starting with cationic C-PAM in 1 mM and 100 mM NaCl on SiO$_2$ and cellulose (Paper III). The figure to the left shows the change in frequency and the figure to the right the change in dissipation. The letters C and A indicate addition of cationic and anionic polyelectrolyte, respectively. f_0=5 MHz, n=3.*

Both high-charge polyelectrolytes (PDADMAC and PESNa) rather evenly increased the change in frequency at both salt concentrations on silica and on cellulose substrates (Figure 5.8, Paper III). Addition of salt significantly increased the Δf especially on SiO$_2$. The change in dissipation remained low, close to zero, at low ionic strength on both surfaces. At high ionic strength the dissipation curves had a zigzag-like form. Addition of cationic PDADMAC increased the change in dissipation, while the addition of anionic PESNa decreased it roughly to the same extent. The polyanion most likely replaces the water in the polycation layer, leading to a more rigid and compact layer (Notley et al. 2005). The final change in frequency and in dissipation obtained using the high-charge polyelectrolytes is much smaller than when using the medium-charge polyacrylamides (Figure 5.7).

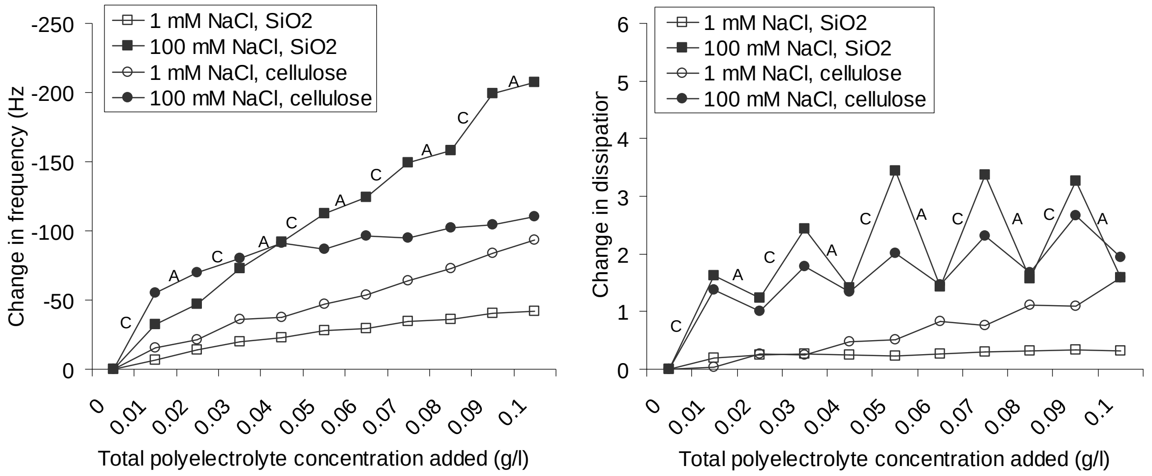


Figure 5.8. *Multilayer formation through sequential additions of 0.01 g/l PDADMAC$_{HC\text{-}LMW}$ and 0.01 g/l PESNa$_{HC\text{-}LMW}$ added at 20-minute intervals, starting with cationic PDADMAC in 1 mM and 100 mM NaCl on SiO$_2$ and cellulose (Paper III). The figure to the left shows the change in frequency and the figure to the right the change in dissipation. The letters C and A indicate addition of cationic and anionic polyelectrolyte, respectively. f_0=5 MHz, n=3.*

The substrate and salt concentration did not significantly affect the adsorption of medium-charge PEMs (Figure 5.7), whereas the high-charge polyelectrolytes adsorbed differently in the multilayering, depending on the ionic strength and surface (Figure 5.8). Medium-charge polyacrylamides formed a more open and viscous multilayer, whereas higher charged polyelectrolytes produced a compact and rigid structure. At high ionic strength the adsorption trend was similar for low- and high-charge PEMs: both polyelectrolytes increased the change in frequency, whereas cationic polyelectrolyte mainly increased the change in dissipation, while the effect of polyanion was negligible or even reducing. The addition of anionic polyelectrolyte causes stiffening of the adsorbed multilayer. Similar periodical variations in the multilayer formation has also been seen for multilayers of polyallylamine hydrochloride (PAH) and polyacrylic acid (PAA) on silica (Notley et al. 2005) and for multilayers of PAH and polysodium 4-styrenesulfonate (PSS) on mica (Kolasińska and Warszyński 2005). The properties of the system seemed to be strongly dependent on the nature of the outermost layer (Eriksson et al. 2005, Notley et al. 2005, Lingström et al. 2006).

5.4.2 Polyelectrolyte complexes

Numerous factors such as the type, charge density and molecular weight of the polyelectrolyte (Mende et al. 2002, Hubbe et al. 2005, Salmi et al. 2007), the ratio between anionic and cationic charges, ionic strength, pH, addition order (Dautzenberg and Jaeger 2002, Chen et al. 2003), agitation (Hubbe et al. 2005) and ageing (Buchhammer et al. 1999, Mende et al. 2002, Chen et al. 2003, Hubbe 2005, Hubbe et al. 2005) have an influence on the complexation, properties of the PECs in solution as well as the adsorption of the complexes. In this chapter the emphasis is in the charge density of the polyelectrolytes, the mixing ratio and ionic strength (Kramer et al. 1998, Pergushov et al. 1999, Dautzenberg 2000, Stenius 2000, Dautzenberg and Jaeger 2002, Mende et al. 2002, Chen et al. 2003, Hubbe et al. 2005) while the other factors have been kept constant.

The complex formation was studied as a function of the mixing ratio by measuring the electrophoretic mobilities of PECs (Paper III). The mobility of the A-PAM/C-PAM complexes decreased gradually when the amount of anionic polyelectrolyte increased in the complex at both measured ionic strengths (Figure 5.9, left). As expected, at high ionic strength the mobility of the A-PAM/C-PAM complexes was lower since in the presence of salt the polyelectrolyte conformation changes from a more or less stiff to a coiled structure due to the screening of the electrostatic repulsion between the charged chains by salt ions.

The mobility of the complexes formed by high-charge polyelectrolytes changed steeply from cationic to anionic at the isoelectric point, which lies near the 1:1 stoichiometric ratio. Low amounts of PESNa in the complex did not change the mobility of the complex since at non-stoichiometric mixing ratios complex particles are electrostatically stabilized (Buchhammer et al. 1999, Mende et al. 2002, Chen et al. 2003, Hubbe et al. 2005). Increasing the mixing ratio up to 1:1 stoichiometry leads to charge compensation between the polyanion and polycation and a secondary aggregation. A slight deviation from the A-PAM:C-PAM stoichiometric ratio in Figure 5.9A could be probably explained by slightly differing molecular weights between polyanion and polycation and steric factors (Buchhammer et al. 1994). In the case of PESNa/PDADMAC complex (Figure 5.9B) the deviation from the 1:1 stoichiometric ratio is due to the slightly differing charge densities between the two polyelectrolytes. At high ionic strength the mobility was lower, as expected, but the complex was still clearly cationic or anionic (Figure 5.9).

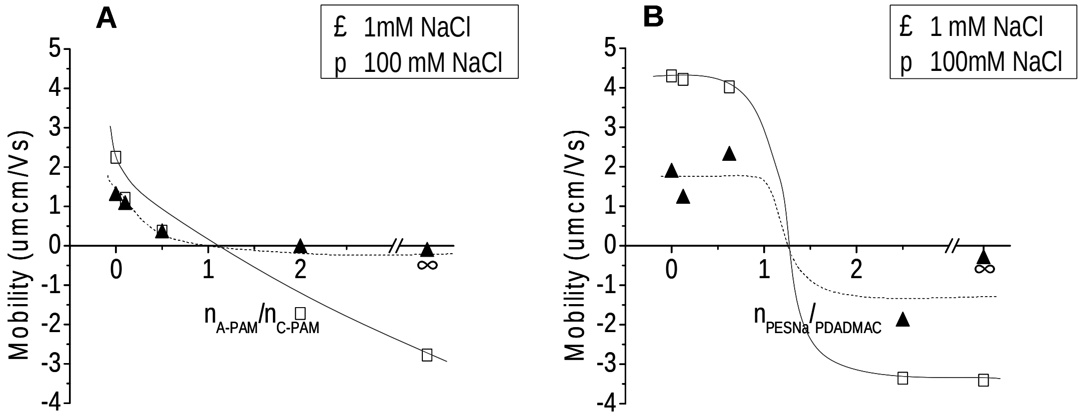


Figure 5.9. *Mobility of the polyelectrolyte complexes formed by A) A-PAM$_{MC\text{-}HMW}$/C-PAM$_{MC\text{-}HMW}$ and B) PESNa$_{HC\text{-}LMW}$/PDADMAC$_{HC\text{-}LMW}$ (Paper III). The concentration of pure polyelectrolytes was 1 g/l and the concentration of PECs varied between 0.1-0.3 g/l.*

The final changes in frequency after adsorption of 0.5 g/l PECs on silica and on cellulose at low and high salt concentrations as a function of charge ratio are shown in Figure 5.10. In this way the effect of ionic strength, the ratio between cationic and anionic polyelectrolytes in the complex, and the surface can be compared. On silica the change in frequency of all complexes formed from polyacrylamides were higher at low ionic strength (open squares). Only C-PAM alone adsorbed more at high salt concentration than at low salt concentration. The charge ratio strongly affected the adsorption at low ionic strength, being the highest at an A-PAM:C-PAM ratio of 0.5 as also found in literature (Kekkonen et al. 2001, Gärlund et al. 2003, Hubbe 2005). At charge ratios near 1.0 the PECs were unstable, as has been found previously (Dautzenberg 1997, Kramer et al. 1998, Pergushov et al. 1999, Stenius 2000, Mende et al. 2002, Gärlund et al. 2007), whereas at the charge ratio of 2.0 the complexes were again stable. Hence, no experiments close to the charge neutralization point were performed, since these would not have been reliable. A substantial adsorption was found at the charge ratio of 2.0 although the complex was clearly negative at this charge ratio, as can be seen in Figure 5.9.

There are two possible reasons for the adsorption in this case. Either the complex, although stable in solution, breaks up close to the surface and only C-PAM adsorbs, or then the complex adsorbs with a conformation were cationic parts of the complex are close to the negative surface as suggested by Gregory (1973, 1976).

In 100 mM NaCl (filled squares in Figure 5.10) the effect of charge ratio of A-PAM/C-PAM complexes was not as pronounced as in 1 mM NaCl. The complexes are still stable at higher electrolyte concentration but most of the electrostatic interactions are screened and the complexes probably have more or less the same conformation at the different charge ratios. The adsorption of the complexes formed by polyacrylamides on cellulose followed the same trends as on silica (open and filled spheres in Figure 5.10). The adsorption of all A-PAM/C-PAM complexes was higher on silica than on cellulose at low and high ionic strength because less polyelectrolyte is needed to neutralise the lower charged cellulose film.

The complexes formed by high charged PESNa and PDADMAC clearly adsorbed at low ionic strength on both silica and on cellulose only at a charge ratio of 0.6 (open squares and open spheres in Figure 5.10 right). Probably the interactions between single PDADMAC and surface and between the complex at the charge ratio of 0.1 and surface were so strong that the surface became immediately covered. The anionic complex (PESNa:PDADMAC=2.5) did not adsorb at all, probably due to the repulsion between like charges on surface and complex. Adding salt changes the conformation of the complex on silica, which can be seen as an increase in Δf at a charge ratio of 0.1 (filled squares). Kekkonen et al. (2001) and Gärlund et al. (2003) have also observed higher adsorbed amounts of complexes formed by strong polyelectrolytes on silica and fibre surfaces at higher ionic strengths. Strong interactions between oppositely charged polyelectrolytes gave very high frequency values for other complexes (PESNa:PDADMAC=0.6 on silica and cellulose and PESNa/PDDAMAC=2.5 on silica), probably as a result of phase separation, i.e. unstable and precipitated complexes, and thus these values are not shown here.

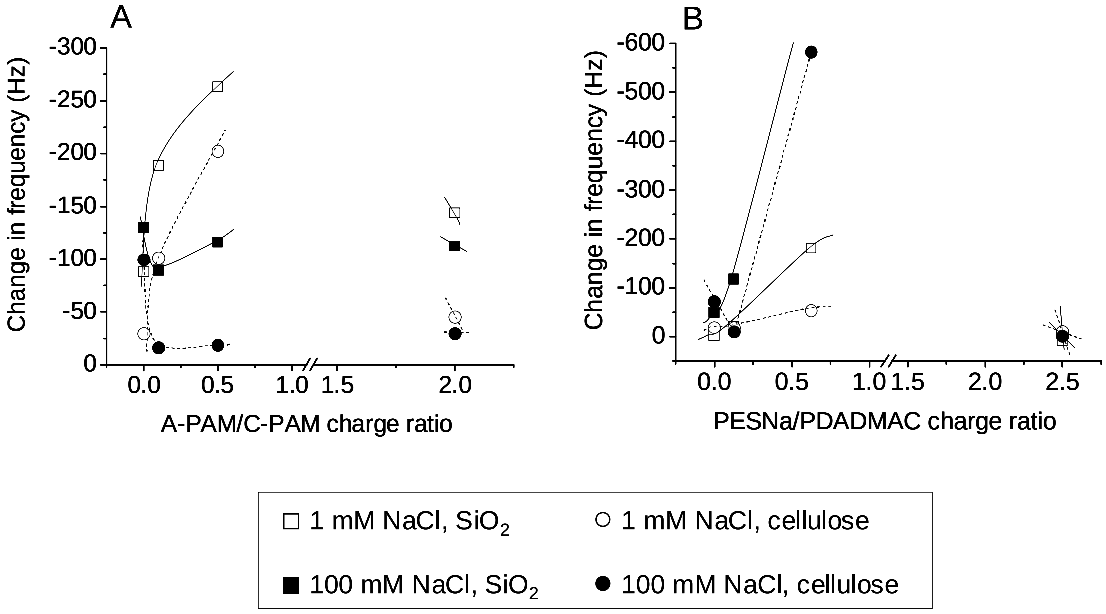


Figure 5.10. *Effect of ionic strength and surface on the adsorption of PECs on SiO_2 and cellulose (Paper III). Change in frequency on adsorption of A) A-PAM$_{MC\text{-}HMW}$/C-PAM$_{MC\text{-}HMW}$ and B) of PESNa$_{HC\text{-}LMW}$/PDADMAC$_{HC\text{-}LMW}$. The added concentration of PECs was 0.5 g/l and the electrolyte concentration 1 or 100 mM NaCl. f_0=5 MHz, n=3.*

The properties of the polyelectrolyte complexes at low ionic strength on SiO_2 and cellulose substrates are presented by plotting the change in dissipation as a function of change in frequency (Paper III). At the beginning of adsorption the *D-f* curves of A-PAM/C-PAM complexes were almost linear, meaning that ΔD increased evenly with Δf on both surfaces (Figure 5.11). The PEC in the charge ratio of 0.5 adsorbed as a very thick and loose layer and as the adsorption proceeded the layer became slightly looser. There are two possible explanations for this: either the structure of the whole adsorbed layer becomes looser or then the latter complexes adsorb in a looser conformation than the first ones. On cellulose the Δf

and *ΔD* of the complexes formed by polyacrylamides were only slightly lower but the curves followed the same trends as that on silica. Only the one-component C-PAM system formed a thicker layer on silica than on cellulose. Surprisingly, the anionic complex (A-PAM:C-PAM=2) adsorbed on both surfaces, probably as a result of patch adsorption, in which the cationic charges in the complex attach to the anionic surface (Gregory 1973, 1976).

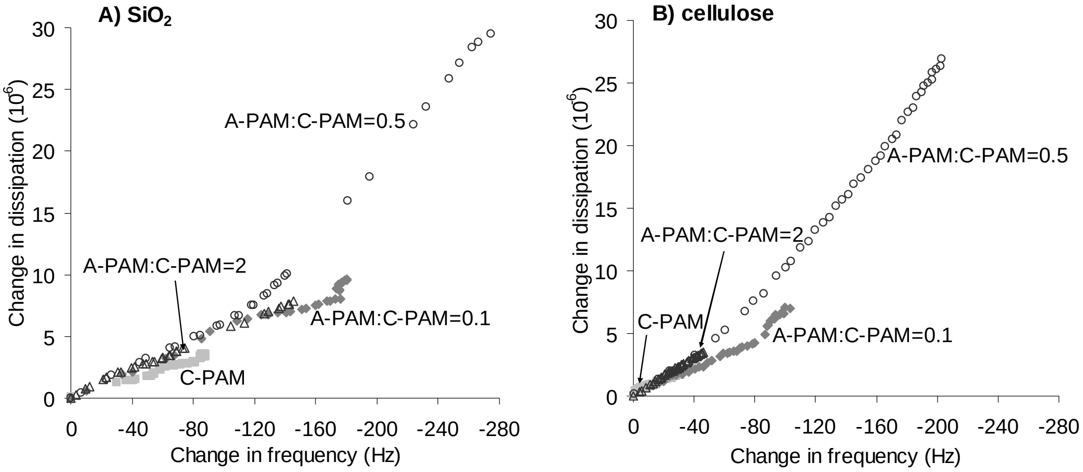


Figure 5.11. *Polyelectrolyte complex isotherm through ten sequential additions of 0.01 g/l premix PEC solution followed by four sequential additions of 0.1 g/l premix PEC solution added at 20-min intervals in 1 mM NaCl on A) SiO_2 and B) cellulose (Paper III). The PEC isotherms were formed by medium-charge* $A\text{-}PAM_{MC\text{-}HMW}$ *and* $C\text{-}PAM_{MC\text{-}HMW}$. $f_0=5$ *MHz, n=3.*

The adsorption of polyelectrolyte complexes was also studied by adding the medium-charge PECs in one addition on cellulose so that the complex concentration was the same as at the end of the complex isotherm in Figure 5.11. The final *Δf* (-238 Hz) and *ΔD* (36×10^{-6}) for the PEC at the charge ratio of 0.5 (unpublished data) seemed to indicate that the way how the PEC was added did not affect the adsorption.

The adsorption of PECs formed by high-charge polyelectrolytes on silica and cellulose is shown in Figure 5.12 (Paper III). The changes in frequency and in dissipation of high-charge and low-molecular-weight PECs were clearly lower than those of medium-charge and high-molecular-weight PECs (Figure 5.11). The PESNa/PDADMAC complexes at the charge ratio of 0.1 and of 2.5 as well as the single cationic PDADMAC immediately covered the high-charge silica surface, which can be seen as a cluster in measurement points. The attraction between PDADMAC and the surface, and between the cationic complex (PESNa:PDADMAC=0.1) and the surface was so strong that the surface became immediately covered. Only at the charge ratio of 0.6 a higher *Δf* was achieved, while the *ΔD* values were still low, indicating a flat layer. In the presence of anionic PESNa the PDADMAC adopts a more coiled conformation and hence the adsorbed amount is higher than for PDADMAC alone (Figure 5.9).

On cellulose the *Δf* of the PDADMAC and the complex at the charge ratio of 0.1 was roughly similar but the complex was more dissipative. The anionic complex (PESNa:PDADMAC=2.5) did not adsorb at all, indicating that the attractive interactions between the low-charge cellulose and PEC were insignificant. Only at the charge ratio of 0.6 the changes in frequency and in dissipation were slightly higher than at the other ratios. The only noteworthy difference between the high-charge complex layers on silica and on cellulose seems to be at the charge ratio of 0.6. At this charge ratio the *Δf* was higher on silica, whereas the *ΔD* was higher on cellulose. This implies that the conformation of the PEC on cellulose was different from that on silica. A clear cationic PEC (PESNa:PDADMAC=0.6) settles in a flat conformation on the high-charge silica whereas on low-charge cellulose a more loose structure was formed.

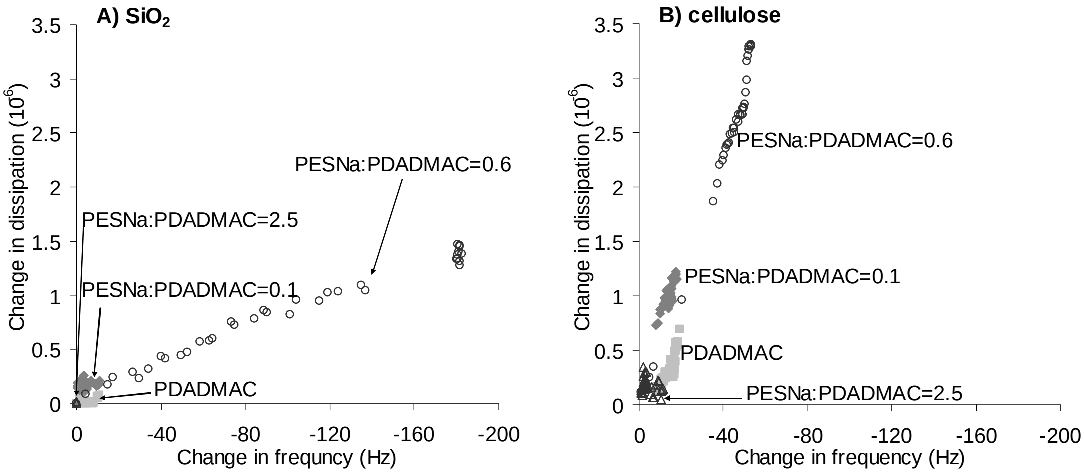


Figure 5.12. *Polyelectrolyte complex isotherm through ten sequential additions of 0.01 g/l premix PEC solution followed by four sequential additions of 0.1 g/l premix PEC solution added at 20-min intervals in 1 mM NaCl on A) SiO_2 and B) cellulose (Paper III). The PEC isotherms were formed by high-charge $PESNa_{HC\text{-}LMW}$ and $PDADMAC_{HC\text{-}LMW}$. f_0=5 MHz, n=3.*

5.4.3 Comparison between single polyelectrolytes, PEMs and PECs

The adsorption of three different systems, single cationic polyelectrolytes, PEMs and PECs at low ionic strength on cellulose is compared in Figure 5.13. The final frequency values after 200 min adsorption of single cationic polyelectrolytes were low on both surfaces and the formed layers were quite rigid. In multilayering the almost linear increase in the Δf and ΔD with an increase in the oppositely charged polyelectrolytes indicated that the adsorbed amount and layer thickness of the PEMs can be increased, in principle, as high as is reasonable to achieve the desired layer properties. The PEMs were quite rigid if compared to the thick and more dissipative PEC layers with higher $\Delta D/\Delta f$ values. Generally, the PESNa/PDADMAC layers were more compact than the A-PAM/C-PAM layers.

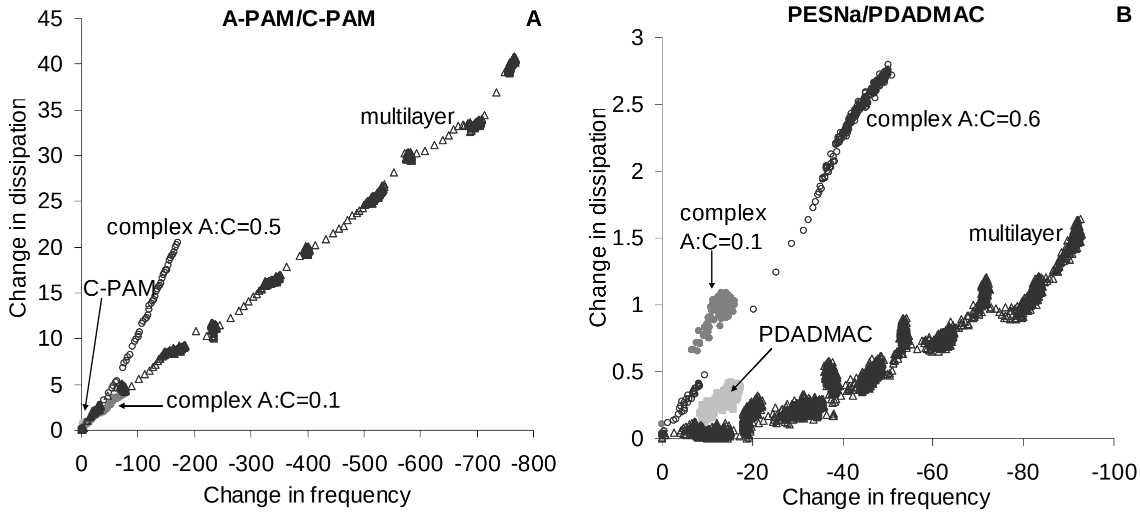


Figure 5.13. *Change in dissipation as a function of change in frequency for adsorption of single cationic polyelectrolyte, polyelectrolyte multilayers and polyelectrolyte complexes formed by A) A-PAM/C-PAM and B) PESNa/PDADMAC in 1 mM NaCl on cellulose (Paper III). The adsorptions were made as isotherms and the final concentration of single polyelectrolytes was 0.1 g/l, of PECs 0.1 g/l, A-PAM/C-PAM multilayer 5 g/l and PESNa/PDADMAC multilayer 0.1 g/l after ten additions.* f_0*=5 MHz, n=3.*

Both systems, PEMs and PECs, have a positive effect on the strength properties of fibres in papermaking. Sequential addition of the polyelectrolytes to the fibre slurry has been found to yield higher strength than premixing the polyelectrolytes before their addition (Hubbe 2005, Hubbe et al. 2005, Heermann et al. 2006). PEM addition enhances also significantly the adhesion between fibres which further increases paper strength (Eriksson et al. 2006, Lingström et al. 2006, Pettersson et al. 2006, Enarsson and Wågberg 2007).

In PEC treatment the increase in paper strength is most likely caused by the increased fibre-to-fibre joint strength (Gärlund et al. 2003, Gärlund et al. 2005, Torgnysdotter and Wågberg 2006, Vainio et al. 2006). Treating the fibres with PECs also improves sheet forming, retention and dewatering (Bessonoff et al. 2006, Xiao et al. 2006), although these properties usually are interdependent, so that improving one impairs the other. The interacting forces are completely different when using complexes instead of single polyelectrolytes or PEMs (Salmi et al. 2007). The complexes formed a looser layer and more extended conformation (long-range interactions) compared to PEMs or single polycations. This may further explain why PECs are effective retention agents. It is evident that the molecular level differences between different polymeric systems found in this study are also reflecting to practical technical applications.

5.5 Modification of cellulose and lignin with laccases

The adsorption studies of two enzymes, *Trametes hirsuta* and *Melanocarpus albomyces* laccases, on cellulose and lignin are summarised in this chapter. First, the cellulose and lignin substrates were modified with laccases at low and neutral pHs. Second, the adsorption of ferulic acid on the laccase-treated lignin was examined. In this case, the adsorption time of laccases was seven minutes and the laccases were added in batch mode, while in the cellulose and lignin activation by laccases the enzymes were added during four hours, using a constant flow of 100 µl/min. The results presented in this chapter are described in more detail in Papers IV and V.

5.5.1 Adsorption of laccases on cellulose and lignin

In laccase-catalysed oxidation of wood fibres, phenoxy radicals are formed in the lignin matrix (Felby et al. 1997). Therefore, laccases are potential enzymes for modification of mechanical pulps which contain a high amount of lignin (Koljonen et al. 2003). Several studies on the action of laccases on isolated lignin have been reported (Barsberg and

Thygesen 1999, Buchert et al. 2002, Rittstieg et al. 2002, Boerjan et al. 2003, Mattinen et al. 2008). No earlier investigations related to enzyme adsorption on a lignin model substrate by QCM has been reported, whereas a few studies on the action of cellulases on cellulose film by QCM-D have been presented (Ahola et al. 2008; Josefsson et al. 2008; Turon et al. 2008).

The adsorption of two laccases, *T. hirsuta* and *M. albomyces*, was studied at two pHs on cellulose and lignin substrates (Figure 5.14). Both laccases adsorbed on both surfaces although according to literature (Kiiskinen et al. 2004) *T. hirsuta* laccase was not able to bind to cellulose. The conformation and the final Δf and ΔD were strongly dependent on the laccase type, pH and the substrate used (Paper IV).

At the beginning of the adsorption both laccases adsorbed rather equally and there was a linear relationship between the Δf and ΔD, suggesting no conformation changes during the adsorption process (Figure 5.14, left). Significantly, the highest change in frequency and in dissipation was obtained when the *T. hirsuta* laccase was added at low pH on lignin. At higher pH the changes in frequency and dissipation values were lower. The Δf also slightly levelled off as the attachment proceeded at pH 7.5, whereas the change in dissipation continued to increase slightly. This suggests a somewhat more viscous laccase layer. On cellulose the final changes in frequency and in dissipation were lower than that on lignin at the corresponding pHs. This is in accordance with theory that laccases strongly interact with lignin (Felby et al. 1997). At the end of *T. hirsuta* laccase adsorption the ΔD levelled off at pH 4.5 on cellulose. This implies a more compact laccase layer.

The pH and substrate strongly affected the behaviour of the *M. albomyces* laccase on cellulose and lignin (Figure 5.14, right). The Δf was high whereas the ΔD was very low at pH 4.5 on lignin, suggesting a flat and compact laccase layer. The increase in the pH noticeably changed the adsorption of the *M. albomyces* laccase. At the beginning the *Δf* was more or less unchanged whereas the *ΔD* strongly increased, suggesting that the adsorbed laccase layer is dissipative and loose at high pH on lignin. This means that a low amount of loosely bound laccase, including coupled water inside the laccase layer, was adsorbed. As the adsorption proceeded the change in frequency started to slightly increase with an increase in the change in dissipation.

On cellulose the adsorption of *M. albomyces* laccase at low pH is similar but clearly lower than the behaviour at high pH (Figure 5.14, right). At the beginning the slope is almost horizontal and the *Δf* slightly increases, whereas the *ΔD* stays more or less constant. As the adsorption proceeds, the change in dissipation starts to increase in relation to the change in frequency, suggesting a more viscous layer.

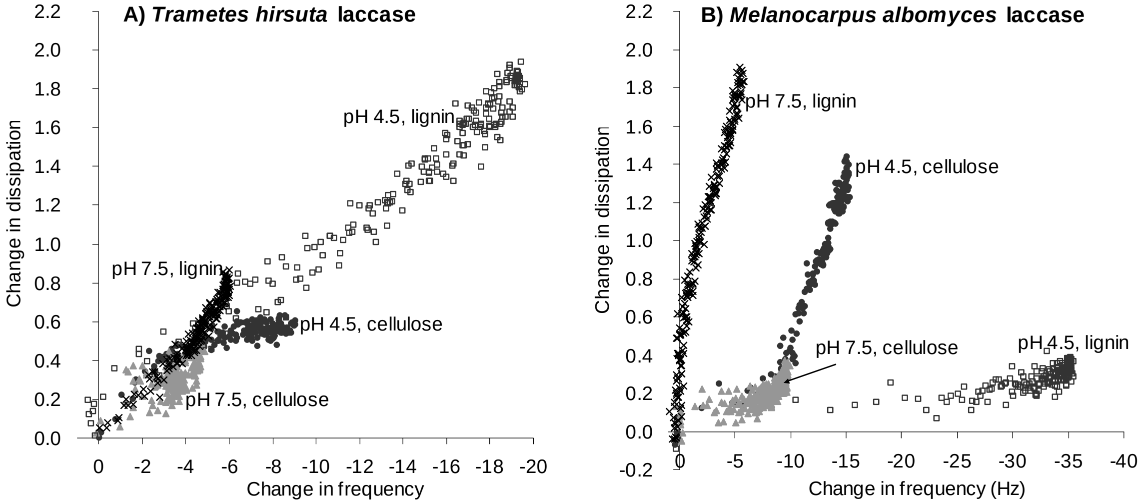


Figure 5.14. *Change in dissipation as a function of change in frequency of the normalized fifth overtone for adsorption of 1 µM A) T. hirsuta and B) M. albomyces laccase at pH 4.5 and 7.5 in 20 mM $NaHCO_3$ on cellulose and lignin (Paper IV).*

In polymeric systems a low *ΔD/Δf* ratio describes compact adsorbed layer and strong interactions between the adsorbed polymer and the surface (Paper II). If the interactions between the adsorbed layer and the surface are weak, more loose structure is formed which can be seen as a higher *ΔD/Δf* ratio. For laccases the interpretation of QCM-D data is more complex due to the complexity of their structures (e.g. three different domains). Hence, if comparing the adsorption of laccase on cellulose and lignin at different pH-levels there are several factors, such as surface charge, charge of laccase, differences in electrically polarized structure, binding site, etc., that are changing with pH. The effects of those individual factors on the adsorption results can not be separated. However, it seems that at low pH there is a high affinity between *M. albomyces* laccase and lignin which leads to a rigid layer whereas at higher pH the interactions are weaker.

Electrostatic and hydrophobic interactions between protein and surface are believed to play a major role in the protein adsorption phenomena (Holmberg 2002). The cellulose substrate has been found to be uncharged at pH 4.5 while at neutral pH the cellulose becomes charged due to an increased number of dissociated carboxylic groups (Österberg and Claesson 2000). For lignin film the pH variations between 4.5 and 7.5 has not been found to affect the charge density (Notley and Norgren 2006). On the other hand, lignin is more hydrophobic than cellulose. The theoretical isoelectric points for *M. albomyces* and *T. hirsuta* laccases are 4.9 and 4.7, respectively (ExPASy ProtParam tool, Gasteiger et al. 2005), meaning that the laccases are negatively charged at pH 7.5. Based on consideration of hydrophobic interactions, the maximum adsorption would occur around the isoelectric point of the protein, and an increase in pH would weaken the hydrophobic interactions (a lower adsorption). The electrostatic repulsion becomes stronger at higher pH, leading into a lower adsorption. Thus, both the electrostatic and hydophobic interactions would explain the higher adsorption of both laccases at pH 4.5 and the differences obtained between the lignin and cellulose surface.

The laccase-treated substrates were also analysed by AFM (Paper IV). All morphological changes were minimal when the laccase-treated surfaces were compared to the non-treated cellulose and lignin substrates. The small size of the enzyme probably makes it difficult to distinguish the adsorbed laccases from the surface features of cellulose and lignin substrates. In addition, the laccases probably settle, following the uneven cellulose and lignin surfaces, which makes the detection even more difficult. The laccase-treatment increases the size of the granules which can also be seen as a more roundish height profiles (Figure 5.15). Treating the surfaces with *M. albomyces* laccase increased also the rms roughness of cellulose (0.8 nm) and lignin (0.9 nm) films if compared to the untreated films (rms roughness 0.4 nm). This kind of behaviour is the opposite to the action of cellulases on cellulose film. The cellulases cause swelling and softening (Josefsson et al. 2008) and a decrease in roughness (Turon et al. 2008) for the cellulose film.

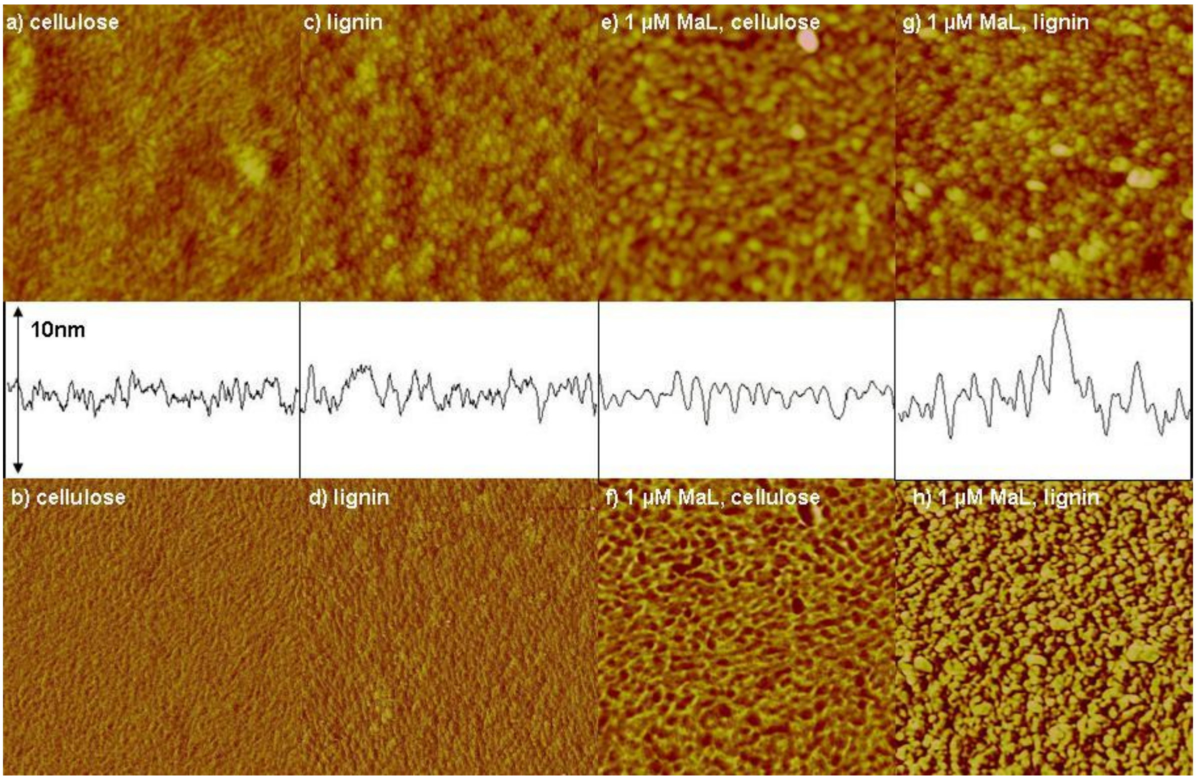


Figure 5.15. *AFM topography (above) and phase contrast (below) images of a-b) pure cellulose, c-d) pure lignin, e-f)1 µM M. albomyces laccase-treated cellulose and g-h) 1 µM M. albomyces laccase-treated lignin substrates at pH 4.5 after 4 h modification and 1 h rinsing (Paper IV). Typical height profiles are shown in the middle. The image size is 1µm^2, all Z ranges are 12 nm in topography images and 12° in phase contrast images.*

5.5.2 Adsorption of ferulic acid on laccase-treated lignin

The activation of fibre surfaces by laccases can be used as a first step in fibre functionalisation when aiming to impart desired properties to fibres via chemo-enzymatic method (Lund et al. 1998, Chandra and Ragauskas 2002, Chandra et al. 2004a,b, Grönqvist et al. 2006). The primary reaction of laccase-catalysed oxidation is the formation of phenoxy radicals on the substrate (Felby et al. 1997). In lignin-rich pulps laccases can be used to activate the surface lignin (Felby et al. 1997; Lund et al. 1998, 2003; Grönqvist et al. 2003), after which specific chemical components can be bound to lignin on the fibre surface in order to tailor the wood fibre properties (Chandra and Ragauskas 2001, 2002, Chandra et al. 2004a,b, Grönqvist et al. 2003, 2006, Fackler et al. 2008). In this thesis the specific chemical component, ferulic acid, was attached to laccase-activated lignin by using two different addition strategies: ferulic acid was added after laccase pretreatment, or coadsorbed together with laccase (Paper V). Hence, the addition of laccase and ferulic acid was made as a multilayer or as a premix complex correspondingly to the adsorption studies of oppositely charged polyelectrolytes.

Pure ferulic acid adsorbed on the native lignin substrate but it desorbed during water rinsing (Figure 5.16). Although no changes in adsorbed amount were observed with *T. hirsuta* laccase after seven minutes' adsorption, both laccases activated the lignin substrate, inducing a higher adsorbed amount of ferulic acid on the laccase-activated lignin than on the pure lignin.

A high amount of adsorption was achieved when *M. albomyces* laccase and ferulic acid were added in a sequence on lignin (Figure 5.16). When the lignin activation was made by *T. hirsuta* laccase, a clearly lower adsorbed amount of ferulic acid was observed. Probably, the addition of ferulic acid removed the unbound *T. hirsuta* laccase but not the adsorbed *M. albomyces* laccase from the system. Rinsing the lignin with water after laccase activation (but before ferulic acid adsorption) significantly decreased the subsequent adsorption of ferulic acid on the lignin substrate. Both unbound *T. hirsuta* laccase and loosely adsorbed *M. albomyces* laccase molecules were apparently rinsed away from the activated lignin. The adsorption of ferulic acid on the laccase-activated, and thereafter rinsed lignin surface suggests that activated lignin can further activate ferulic acid that can thereafter attach on lignin by a radical mechanism. On the other hand, when the lignin was not rinsed after laccase treatment the excess laccase could probably also polymerise the ferulic acid and further increase the adsorbed mass. Similar attachment of tyramine to laccase-treated wood fibres has also been reported (Grönqvist et al. 2006).

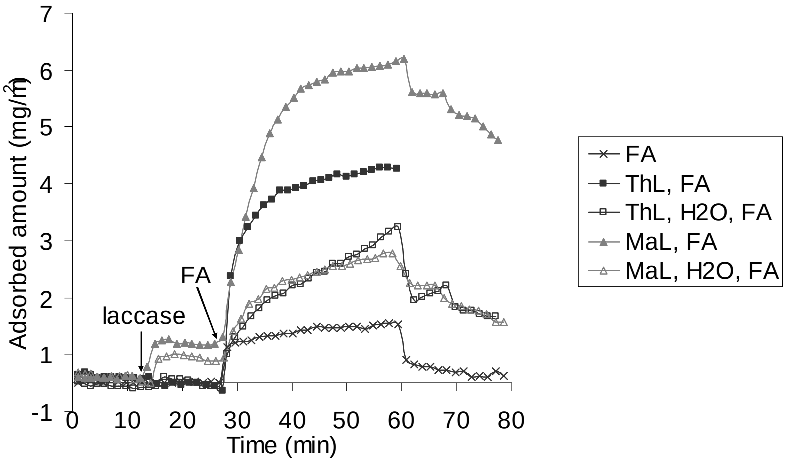


Figure 5.16. *Change in adsorbed amount calculated from Eq. 2 as a function of time for adsorption of T. hirsuta (ThL) and M. albomyces (MaL) laccases (21 µg protein/ml) and ferulic acid (FA, 1 g/l), added one after the other, at pH 4.5 on lignin (Paper V). Laccases were added after 15 min and ferulic acid after 27 min. Water rinsing was made once after enzyme addition (22 min) and three times after ferulic acid addition (60, 68 and 74 min).*

Ferulic acid and laccase were also added as a premixed complex on lignin (Figure 5.17, Paper V). When the ferulic acid and *T. hirsuta* laccase were added together either straight on the untreated lignin or on the laccase-activated lignin, the differences between the adsorbed amounts were minimal. However, the rate of adsorption was clearly lower if the substrate was not activated by laccase in advance or if the lignin was rinsed after laccase activation. The clear adsorption of ferulic acid on non-activated lignin shows that *T. hirsuta* laccase could simultaneously catalyse the lignin substrate and boost the adsorption of ferulic acid.

Similar ferulic acid adsorptions were made using the other enzyme, *M. albomyces* laccase (Figure 5.17 right). The highest adsorbed amount of ferulic acid was found for laccase-activated lignin without a rinsing step. Rinsing of excess laccase prior to addition of ferulic acid solution decreased the adsorbed amount of ferulic acid. Probably in this case the *M. albomyces* laccase polymerised only the ferulic acid (Carunchio et al. 2001) but could not activate the lignin. This prevents strong attachment of ferulic acid on lignin. When the premixed complex solution of laccase and ferulic acid was added on the non-activated lignin, the adsorbed amount was approximately the same as the adsorbed amount of pure ferulic acid (Figure 5.16). Both these ferulic acid layers were rinsed away during water rinsing.

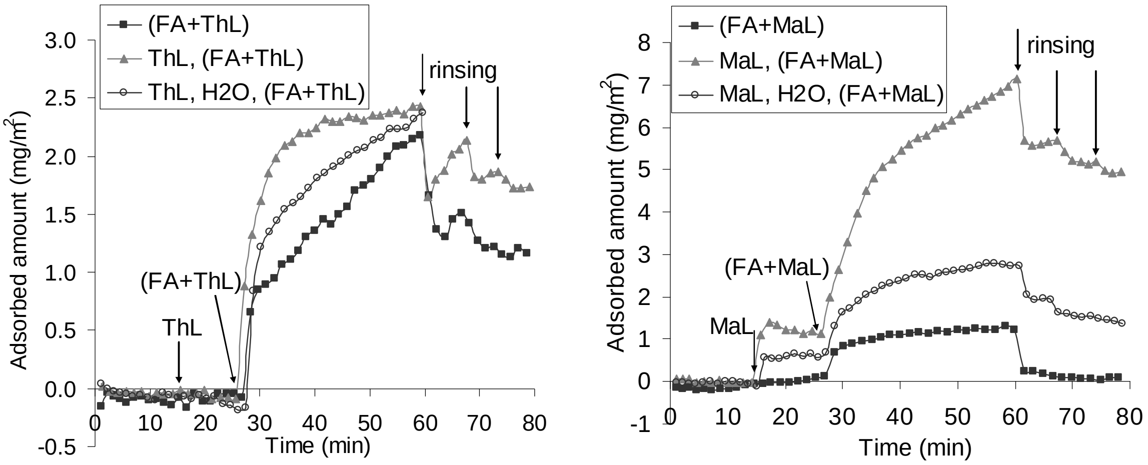


Figure 5.17. *Change in adsorbed amount calculated from Eq. 2 as a function of time for adsorption of T. hirsuta (ThL, left) and M. albomyces laccase (MaL, right) and ferulic acid (FA) added together at pH 4.5 on pure lignin or laccase-activated lignin (Paper V). Laccase (21 µg protein/ml) was added as such after 15 min and FA+laccase (1 g/l FA, 0.042 µg protein/ml laccase) after 27 min. Water rinsing was made once after laccase addition (22 min) and three times after ferulic acid addition (60, 68 and 74 min). Note the different scales.*

There seems to be a clear difference in lignin activation between *T. hirsuta* and *M. albomyces* laccase. When the ferulic acid and laccase were added together on a native lignin, *T. hirsuta* laccase can activate the lignin substrate together with the polymerisation of ferulic acid, leading to a stable attachment of ferulic acid. On the other hand, *M. albomyces* laccase results only in the polymerisation of ferulic acid without any attachment to lignin. The reason for the different behaviour of the two laccases is not clear.

The adsorption of ferulic acid and ferulic acid-laccase solution on the laccase-activated lignin is illustrated in the AFM images in Figure 5.18. Circular shapes, probably polymerised ferulic acid, are easily seen in the images in which the adsorbed amount of ferulic acid was also high. The amount of circular shapes was lower when the ferulic acid was added on *T. hirsuta* laccase-activated lignin than if the lignin was oxidised with *M. albomyces* laccase (Figure 5.18 a-d). The addition of enzyme and ferulic acid together on laccase-activated lignin is shown in Figures e-h. Plenty of circular shapes are formed on the lignin substrate if the ferulic acid was added together with *M. albomyces* laccase. The rms roughness was also high when clear circular shapes could be seen (Figure 5.18 a-b, g-h).

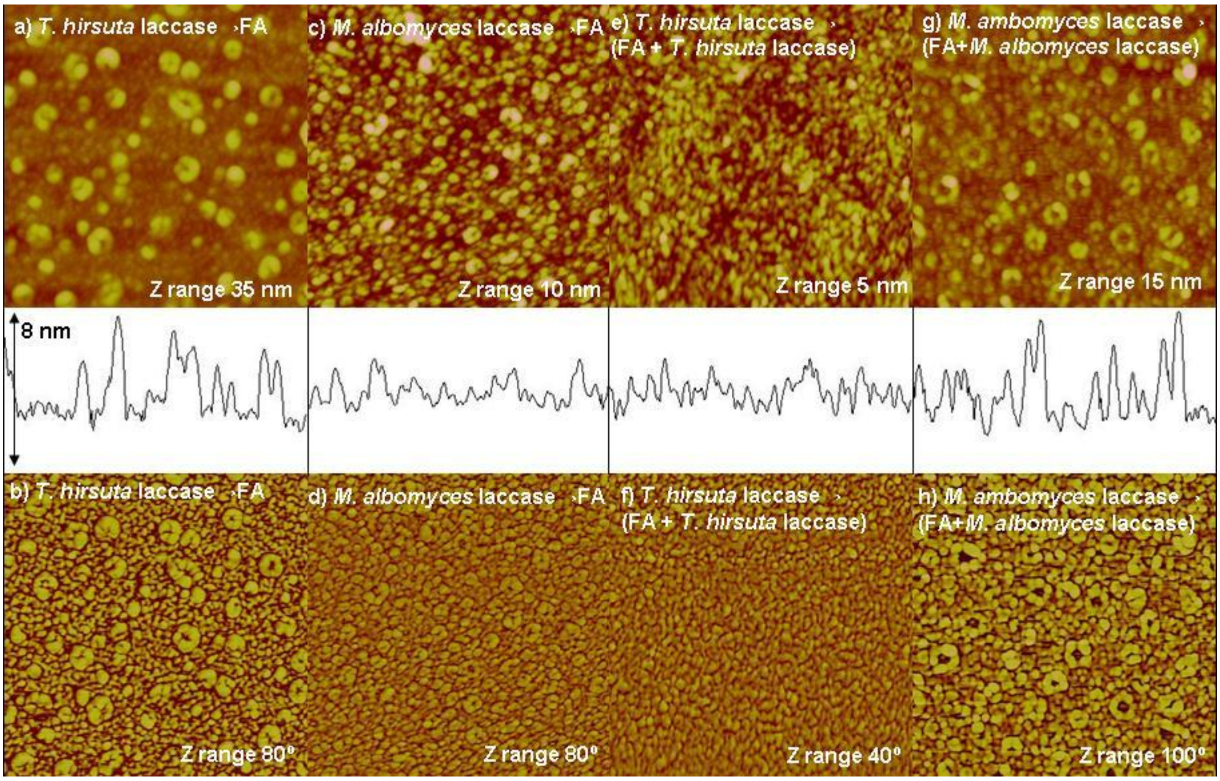


Figure 5.18. *AFM topography (above) and phase contrast (below) images of lignin substrates when a-d) enzyme (21 µg protein/ml) and ferulic acid (FA, 1 g/l) were added one after the other and when e-h) enzyme (0.042 µg protein/ml laccase) and FA were added together (Paper V). a-b) FA was added on T. hirsuta laccase-activated lignin, c-d) FA was added on M. albomyces laccase-activated lignin, e-f) FA and T. hirsuta laccase were added together on T. hirsuta laccase-activated lignin, g-h) FA and M. albomyces laccase were added together on M. albomyces laccase-activated lignin. Typical height profiles are shown in the middle. The image size is* $1\mu m^2$.

6 CONCLUDING REMARKS

The QCM-D technique has been found to be a useful tool for studying the adsorption of various polymeric and enzymatic systems on solid surfaces. QCM-D analysis is rather simple and sensitive and it offers possibilities to elucidate the conformation and viscoelastic properties of the adsorbed layer. Taking into account the coupled water inside the adsorbed layer might be a disadvantage which, however, can be eliminated by using deuterated water during the QCM-D tests or by combining other instruments with the QCM-D technique. On the other hand, measurements in aqueous conditions enable studying the swelling and viscoelasticity of the adsorbed layer.

The charge density of the polyelectrolyte and substrate, as well as the ionic strength strongly affected the adsorption of polyelectrolytes. The low-charge polyelectrolytes adsorbed more on high-charge silica while the adsorption of high-charge polyelectrolytes was higher on low-charge cellulose. On cellulose the adsorption kinetics was clearly slower than on silica. At the beginning of the adsorption the polyelectrolyte films were viscous and more dissipative on cellulose and as the adsorption proceeded the layers became more rigid and stiffer, in contrast to the behaviour on silica. In addition, the differences in layer thicknesses between the different polyelectrolytes were not as pronounced at low ionic strengths on cellulose as on silica. It means that both chemical environment (pH, salt), properties of polycation (charge density, molecular weight) and properties of solid surface (porosity, charge density) influence significantly the extent of adsorption and layer properties of adsorbed polyelectrolytes.

When adding sequentially oppositely charged polyelectrolytes on the silica and cellulose both the change in frequency and in dissipation increased quite linearly at low salt concentration. A higher adsorbed amount (*Δf*) was obtained using low-charge polyelectrolytes for multilayering compared to high-charge polyelectrolytes. This is partly due to the fact that the amount of coupled water is higher for PEMs using low-charge polyelectrolytes. In practise, this has been found to play a significant role in the function of papermaking additives. The substrate and electrolyte did not affect the low-charge PEM adsorption, whereas an increase in salt concentration increased the adsorption of high-charge PEMs. This is mainly due to the fact that the conformation of high-charge polyelectrolytes changes significantly when adding salt, i.e. the conformation changes from flat to more coiled, hence, increasing the total adsorbed amount.

The solution properties of PECs are strongly dependent on the charge density of the used polyelectrolytes, the charge ratio between polycation and polyanion and ionic strength of the solution. The conformation of the PECs in solution will obviously affect their adsorption on solid surfaces. In this study the highest changes in frequency were obtained at the anionic/cationic charge ratio of 0.5-0.6. The complexes formed by low-charge polyacrylamides adsorbed more on silica while the adsorption of high-charge PEC was higher on cellulose, similar to the single cationic polyelectrolytes. The low-charge complexes were more dissipative and viscous than the high-charge PECs. Generally, the PEMs and PECs formed by low-charge polyacrylamides adsorbed highly and formed rather thick layers on both surfaces, unlike the high-charge polyelectrolytes which formed flat and thin layers with both addition techniques. Using polyelectrolyte complexes instead of the multilayering technique thick and dissipative layers with a lower amount of polyelectrolytes can be formed. However, these findings need further clarification and practical experiments before they can be used for developing addition strategies for industrial applications.

Considering the adsorption experiments of polyelectrolytes critically, some additional analysis e.g. with ESCA and AFM could have shed more light on the surface chemistry and morphology of different polyelectrolyte films. Furthermore, a comparison of the results e.g. to ellipsometric studies could have assisted the determination of the amount of coupled water inside the polyelectrolyte layers. Examining the papermaking properties of handsheets made from polyelectrolyte-, PEM- and PEC-treated fibres as well as flocculation measurements when using different addition strategies would also have provided useful information. In addition, the adhesion (interactions forces) between various polyelectrolyte systems and lignocellulosic model surfaces could have been studied, e.g. with AFM. Such studies are ongoing in our laboratory.

The adsorption of *T. hirsuta* and *M. albomyces* laccases on cellulose and lignin substrates was also studied by QCM-D and by AFM. As expected, laccases adsorbed less on cellulose than on lignin. Generally, the adsorbed amounts of *T. hirsuta* laccase were lower than that of *M. albomyces* laccase. The behaviour of *M. albomyces* laccase on lignin was highly dependent on the pH used. At low pH a compact laccase layer was formed on lignin whereas at neutral pH the adsorbed laccase layer was viscous and loose.

Chemo-enzymatic adsorption of ferulic acid on laccase-modified lignin was also studied. A stepwise addition of laccase and ferulic acid achieved a stable attachment of ferulic acid on lignin in contrast to the non-stable ferulic acid adsorption on a non-activated surface. *T. hirsuta* laccase could activate both the ferulic acid and the lignin when they were added as a complex, achieving a stable attachment of polymerised ferulic acid, unlike the *M. albomyces* laccase which probably activated only the ferulic acid. The polymerisation of ferulic acid by laccases could be seen as circular shapes by AFM.

QCM-D and AFM studies gave new information on the attachment of laccases on cellulose and lignin model surfaces. Further research is, however, needed to explore the action of laccases in fibre modification. Combining the QCM-D and AFM e.g. with a surface sensitive chemical technique (ESCA, ToF-SIMS) would provide supplementary information about the action of laccases on fibre surfaces. The effect of laccases on process conditions in papermaking and the papermaking properties of laccase-treated handsheets would also be interesting to examine in connection with the adsorption studies.

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ISSN 1797-4496

ISBN 978-951-22-9653-8

ISSN 1797-5093 (PDF)

ISBN 978-951-22-9654-5

Picaset, Helsinki 2008