LACCASE AIDED MODIFICATION OF NANOFIBRILLATED CELLULOSE WITH DODECYL GALLATE

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Nanofibrillated cellulose, NFC, is an interesting wood fibre-based material that could be utilized in coatings, foams, composites, packages, dispersions, and emulsions, due to its high tensile strength and barrier properties, light weight, and stabilizing features. To improve applicability and properties of NFC, modification of its surface properties is often needed. In this study, the applicability of laccase-aided surface modification with hydrophobic dodecyl gallate (DOGA) on unbleached NFC was investigated. Also, laccase-catalyzed polymerization of DOGA and other phenolic compounds with lignin moieties was investigated by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS). NFC modified with *T. hirsuta*-based laccase and DOGA showed decreased hydrophilicity, as compared with the native NFC, when coated on a paper surface. When dried as free-standing films, the surface properties of chemo-enzymatically modified NFC resembled those of the native NFC. The effect of modification was thus greatly influenced by different surface formation in differently prepared samples. Also, changing of the dispersion properties of DOGA by enzymatic polymerization affected the surface properties of the dried NFC samples. Covalent bonding between DOGA and NFC was not the main factor affecting the surface properties of the NFC in free-standing films or coatings.

Keywords: Nanocellulose; Laccase; Grafting; Dodecyl gallate; Contact angle; Matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS)

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INTRODUCTION

Interest in different nanoscale materials has intensively expanded during the past few years due to their superior physico-chemical properties and wide applicability. Nanofibrillated cellulose (NFC) from wood fibers has recently raised wide interest due to its unique properties applicable in the areas of coatings, foams, composites, packages, dispersions, and emulsions. NFC is composed of liberated cellulose microfibrils, which are the basic structural components of wood fibers. Aqueous suspensions of cellulose microfibrils below 100 nm in diameter behave as gels (Turbak *et al.* 1983; Pääkkö *et al.* 2007). Water suspensions of fibrils can be prepared from wood fibers by liberating the fibrils, *e.g.* by the mechanical shearing in a high pressure homogenizer as described by Turbak *et al.* (1983). Mechanical fibrillation has usually been carried out for bleached pulps, but unbleached kraft pulps can also be nanofibrillated. Unbleached kraft pulps

contain residual lignin bound by physical and chemical interactions, such as hydrogen bonds, Van de Waals' forces, and covalent bonds, to the carbohydrate polymers of fibers (Stenius 2000).

Laccases are blue-copper enzymes that catalyze the oxidation of a variety of phenolic compounds such as dihydroxybenzenes, substituted phenols, and polyphenols, as well as phenolic amines and lignin. In laccase-catalyzed oxidation, a phenolic hydroxyl group loses a single electron and a phenoxy radical is formed (Thurston 1994). Due to the high reactivity of these radicals (either with each other or with a secondary substrate), further reactions, such as polymerisation, depolymerisation, co-polymerisation and grafting, can occur. Laccase-catalysed formation of radicals has been exploited in the activation and the further polymerisation or grafting of lignin, lignans, or small molecular weight lignin model compounds with fibre-bound lignin (Felby *et al.* 1997; Lund *et al.* 1998; Hüttermann *et al.* 2001; Lund and Ragauskas 2001; Chandra and Ragauskas 2002; Grönqvist *et al.* 2003; 2005; Saarinen *et al.* 2009; Suurnäkki *et al.* 2010). In chemo-enzymatic derivatization, completely new chemical functionalities have been grafted onto the lignin-containing fibres (Grönqvist *et al.* 2006; Suurnäkki *et al.* 2006). Laccase-catalysed modification has successfully been applied with other lignocellulose materials using various phenolic and polyphenolic compounds as modifiers, which has been extensively reviewed by Kudanga *et al.* (2011). In the previous studies, materials such as thermomechanical pulps, kraft pulps, wood chips, flax fibers, handsheets and many other macroscopic materials have been utilized as substrates for enzymatic modifications. This study, however, is the first in which laccase-catalyzed modification is used for NFC valorisation.

Surface modification of NFC is often needed to improve its functionality in different applications, such as coatings, foams, composites, packages, dispersions, and emulsions. For example, NFC is not compatible with non-polar substances due to its hydrophilic chemical nature. Reactive hydroxyl groups of the cellulose chain offer many possibilities to different chemical modifications, as has been extensively studied over the decades. Typical chemical modifications of cellulose include substitution reactions of cellulose hydroxyl groups by esterification or etherification in which the original cellulose properties, such as solubility, can be drastically changed by the new functional groups. Ionic or radical grafting of monomeric or polymeric molecules onto the cellulose backbone can be used when the intrinsic properties of cellulose need to be retained (Isogai 2001; Heinze and Liebert 2001; Roy *et al.* 2009). Some chemical modification methods aiming to decrease the hydrophilicity of cellulose chain have been applied with fibrillated cellulose. For example, different substitution methods have been applied, *inter alia*, by Goussé *et al.* (2004a) , Andresen *et al.* (2006a), and Pahimanolis *et al.* (2011a), and grafting methods by Stenstad *et al.* (2008) and Littunen *et al.* (2011). Chemical modification methods, however, often have some drawbacks, such as the need of water removal prior to chemical reactions and harsh reaction conditions leading to partial solubilization of microfibrils and decreased degree of polymerization (DP) of cellulose chains leading to inferior strength properties. These drawbacks can be avoided by exploitation of enzymatic modification methods, which are conducted in water solutions under mild conditions.

Formation of covalent coupling between modifying chemical and isolated or fibre-bound lignin by laccase-catalyzed oxidation has been evidenced by different analytical methods. Laccase-catalyzed coupling of 4-hydroxyphenylacetic acid to kraft

lignin have been confirmed by NMR-spectroscopy (Lund and Ragauskas 2001). Coupling of phenolic amines, fluorophenols, and wood preservatives onto lignin model compounds, such as dibenzodioxocin, guaiacylglycerol *β*-guaiacyl ether (EROL), and syringylglycerol *β*-guaiacyl ether, by laccase have been demonstrated by high performance liquid chromatography (HPLC), liquid chromatography equipped with mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy (Kudanga *et al.* 2009; 2010a; 2010b). In addition, chemical bond formation by laccase has been evidenced between a secoisolariciresinol lignan and ferulic acid by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) by Mattinen *et al.* (2009).

The objective of this study was to modify surface properties of lignin containing NFC by a chemo-enzymatic derivatization method using the high redox potential *Trametes hirsuta* laccase (ThL) and dodecyl gallate (DOGA) as the derivatizing agent. DOGA has previously been utilized as a model compound in laccase-aided hydrophobization of thermomechanical pulp (TMP) (Suurnäkki *et al.* 2006). In addition, DOGA has been more recently used for modification of wool fabric to improve its hydrophobic, antimicrobial, and antioxidant properties (Gaffar Hossain *et al.* 2009; 2010). In this study, covalent binding of the aromatic ring of DOGA to the aromatic ring of two other phenolic lignin model compounds, propyl galate (PROGA), and EROL, by laccase-catalysed oxidation was examined by MALDI-TOF MS. Thereafter, unbleached and bleached NFC was treated with laccase and DOGA; the success of this enzymatic modification of NFC was evaluated by contact angle (CA) measurements of free-standing films and paper coatings. The effects of the treatments on the mechanical properties of the materials were evaluated by tensile measurements of free-standing films. Topography of the modified surfaces was visualized with a surface profiler.

EXPERIMENTAL

Fibre Materials, Processing, and Characterisation

Nanofibrillated cellulose (NFC) made of unbleached and fully bleached birch kraft pulps were used in this study. The pulps were delivered from a Finnish pulp mill (UPM-Kymmene Oyj, Finland). Unbleached pulp was obtained from kraft pulping process before the oxygen delignification stage. Chemical composition of the kraft pulps is presented in Table 1.

Table 1. Chemical Composition of Pulp Materials

Pulp	Lignin i.[a] (% of d.w.)	Lignin s.[b] (% of d.w.)	Lignin total (% of d.w.)	Extr.[c] (% of d.w.)	Glucans (% of d.w.)	Xylans (% of d.w.)	Mannans (% of d.w.)	Mono-sacch. tot. (% of d.w.)
Bleached	n.d.	n.d.	n.d.	n.d.	74.7	25.6	1.0	101
Unbleached	5.0	0.6	5.6	0.6	74.2	20.7	1.1	96

[a] insoluble; [b] soluble; [c] Acetone extractives; nd=not detected

Nanofibrillation of the pulps was carried out with a high-pressure fluidizer (Microfluidics M700, Microfluidics Int. Co., Newton, MA, USA) at VTT. Prior to fibrillation, the never-dried kraft pulps were subjected to pre-refining in a laboratory-scale refiner at a consistency of 4% (Voith Labrefiner LR-1, Voith) using a nominal energy consumption of 300 kWh/t. Then the pulps were diluted with distilled water to a consistency of 1.8% and passed once and then six and ten times through a chamber pair of 500 μm + 200 μm and 200 μm + 100 μm, respectively (unbleached 6 passes, bleached 10 passes). The operating pressure applied was 1850 bars. The nanofibrillated material was stored as such at +4 ºC until used.

Rheological measurements were carried out to analyse the viscoelastic properties of the nanofibrillated material. Small deformation oscillatory stress sweep tests were carried out with a stress-controlled rheometer (AR-G2, TA Instruments, UK) equipped with a vane geometry (vane radius 14 mm and height 42 mm; cup radius 15 mm). The frequency was 1 Hz, and the stress ranged from 0.1 to 1000 Pa. The sample was gently mixed with a spatula before introducing it into the rheometer cup. About 40 mL of sample was spooned into the measuring cup, and the vane was manually set to the measuring position so that the vane was just immersed (gap about 20 mm). Due to the observed time dependency of the samples, the measurement was started exactly 2 min. after the vane was lowered to the measuring position.

The amount of fine nanoscale material in NFC was analysed utilizing a method to separate large-size fibril aggregates from finer nanoscale material as described by Ahola *et al.* (2008a,b). The diluted nanofibril gel (1.7 g L^{-1}) was sonicated for 10 min. with a micro tip at 25% amplitude (Digital Sonifier Model 450 with microtip model 102C, Branson Ultrasonics Co., Danbury, Connecticut, USA) and centrifuged at 10000 g (Eppendorf Centrifuge 5804 R, Eppendorf AG, Hamburg, Germany) for 2 h. After centrifugation, the clear supernatant was removed by pipette and dried for determination of dry solids content. The material present in the clear supernatant was considered as nanoscale material.

Lignin content of unbleached NFC was analysed by isolating acid-insoluble lignin using the Klason method (TAPPI Test Method T 222, 1983). The wavelength used in the spectrophotometric assay of soluble lignin was 205 nm; an absorptivity coefficient 110 L g^{-1} cm^{-1} (Dence and Lin 1992) was used in the quantification of soluble lignin. Carbohydrate composition of the pulps was determined after sulphuric acid hydrolysis (Puls *et al.* 1985) by Dionex CS-3000 gradient HPLC system (Dionex 10 ICS-3000, Sunnyvale, CA) using a CarboPac PA-1 column (Dionex, Sunnyvale, CA).

Chemicals

Dodecyl gallate, DOGA (Dodecyl-3,4,5-trihydroxybenzoate, MW 338.44 g mol^{-1}, exact mass 338.2093 Da), was purchased from Merck Schuchardt OHG (Hohenbrunn, Germany) and used as a dispersion in NFC modifications. A DOGA dispersion (0.024 mol L^{-1}) was prepared according to Buchert *et al.* (2007) using dispersing agents Fennodispo A41 (Kemira Oyj, Espoo, Finland) and lecithin (*i.e.* l-α-phosphatidylcholine) from egg yolks (Fluka Chemie GmbH, Buchs, Switzerland) The dispersion was kept warm at 60 °C prior to use in the experiments. Propyl galate, PROGA (MW 212.2 g mol^{-1}, exact mass 212.0685 Da) was purchased from Acros Organics (New Jersey, USA). Guaiacylglycerol β-guaiacyl ether, EROL (MW 320.337 g mol^{-1}, exact mass 320.1260 Da) was synthesized according to the procedure described by Sipilä and

Syrjänen (1995). The structures of the molecules are shown in Fig. 1. Other chemicals used in the study were of analytical grade.

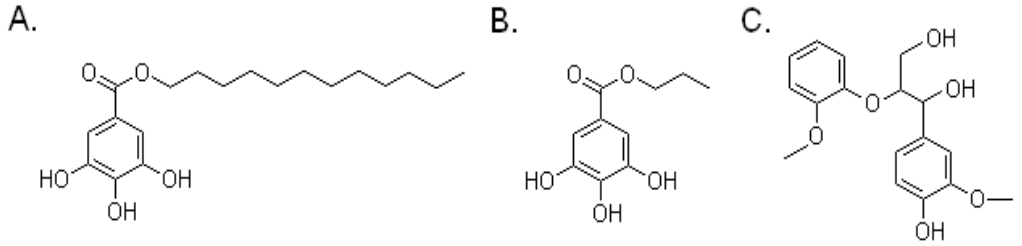


Fig. 1. Structures of model compound (A) DOGA, (B) PROGA, and (C) EROL

Enzyme

Trametes hirsuta laccase (ThL) was produced and purified at VTT according to Rittstieg *et al.* (2002). The activity of the enzyme was determined using the method described by Niku-Paavola *et al.* (1988) using ABTS (2,2′-azinobis[3-ethylbenzthiazoline-6-sulfonic acid]) as a substrate in 4.7 mM concentration in 25 mM succinic acid buffer at pH 4.5 and at room temperature. Catalytic activity was expressed as nanokatals (nkat). One katal is defined as the amount of enzyme activity that converts 1 mol of substrate per second under the assay conditions.

Reactivity of NFC and Model Compound and Functional Chemicals with ThL

The reactivity of bleached and unbleached NFC, as well as selected small-molecule chemicals with ThL was verified by monitoring the consumption of dissolved oxygen after laccase addition by an Orion Sensor link PCM 800 potentiometric electrode (ATI Orion, Boston, USA) as described previously (Mattinen 2008, 2009). Measurements were carried out at pH 4.5 at room temperature, except in the case of DOGA, for which the reaction was conducted at 45 °C to diminish aggregation of the compound. NFC measurements were conducted at 1% (w/w) consistency using ThL dosage of 500 nkat per gram of the oven dry NFC. Concentrations of DOGA, PROGA, and EROL were 1.2 mM and ThL dosage was 0.2 nkat per μmol of substrate. The reactivity of EROL with ThL was determined in 10% acetone solution to improve solubility of the compound. Before the addition of ThL, samples were stabilized at room temperature for 40 min. However, in the case of DOGA, ThL was added prior to the chemical to avoid aggregation. The measurements were carried out under constant mixing in 32 mL volume in fully sealed flasks to avoid entry of oxygen in the vessel during the measurement.

Enzymatic Treatments of Model Compounds and Functional Chemicals

Reaction kinetics analyzed by HPLC

Reaction kinetics of ThL-treated EROL and PROGA was studied by HPLC (Agilent 1200, Agilent Technologies, Santa Clara California, USA). For the analyses, EROL was dissolved in acetone and diluted to 10 mM with 25 mM succinic acid buffer (pH = 4.5) and incubated with ThL (10 nkat μmol^{-1}) for 2 min. prior to the addition of PROGA. The molar ratio of EROL and PROGA was 1:1 in the reaction, which was carried out at room temperature. The oxidation of EROL and PROGA alone by ThL was also followed under similar reaction conditions for comparison. Aliquots of 100 μL were

taken from the reaction mixtures after 2, 5, 15, 30, 60, and 120 min., as well as 24 h incubation times and mixed with 400 μL of a 50% methanol-water solution (v/v), 1 mM sodium azide, and 1.2 mM ascorbic acid. The samples were filtered through 0.45 μm Acrodisc GHP Membrane HPLC filter (Waters, Milford MA, USA). A HPLC auto sampler was used for injection of 5 μL of the samples into the HPLC column. Chromatographic separation was carried out with a reverse-phase Zorbax Eclipse XDB-C18 (2.1 x 100 mm, 3.5 μm) column. A diode array detector was used for detection at a 270 nm wavelength. Chromatographic elution was carried out using five minutes isocratic elution with a 50% methanol-water eluent (v/v) with a 0.15 mL $min.^{-1}$ flow, followed by ten minutes gradient from 50% methanol to 100% methanol with a 0.15 mL $min.^{-1}$ flow, five minutes 100% methanol with a 0.2 mL $min.^{-1}$ flow (to wash the column), five minutes gradient from 100% methanol to 50% methanol with a 0.15 mL $min.^{-1}$ flow, and finally five minutes equilibration of the column to starting conditions with a 0.15 mL $min.^{-1}$ flow.

Polymerization analyzed by MALDI-TOF MS and SEC

Polymerization of PROGA and DOGA, as well as formation of heterocompounds between modifying chemicals and lignin model compound (EROL) after ThL treatment were studied with a Bruker Autofex II MALDI-TOF MS (Bremen, Germany) and by size-exclusion chromatography (SEC).

Oxidation of DOGA by ThL was carried out under similar reaction conditions as NFC treatments. DOGA was added in small batches into a ThL-containing reaction mixture (pH 4.5) at a constant temperature of 45 °C. Enzymatic reactions were carried out in an open flask under constant stirring for 90 min. The action of ThL was terminated by addition of 50 μL 0.05% (w/v) sodium azide (NaN_3). Substrate concentration was 1.2 mM and ThL dosage was 4 nkat per μmol of substrate. In addition to MALDI-TOF MS analysis, SEC was used to verify polymerization of DOGA using the method described by Mattinen *et al.* (2009; 2008).

Coupling reaction of EROL and PROGA by ThL for MALDI-TOF MS analysis was performed as for HPLC analyses, except that during the coupling reaction, concentration of EROL was 4 mM and concentration of PROGA was 2 mM. The smaller amount of PROGA was justified by its better reactivity with ThL, as evidenced by HPLC. After 2.5 h incubation time, the reaction was terminated by addition of 4 μL0.05% NaN_3.

If a precipitate was formed during the reaction time, it was washed with water, frozen, and dried in a vacuum drier (Christ Alpha 2-4, B. Braun Biotech International GmbH, Melsungen, Germany) prior to analysis by MALDI-TOF MS. For the MALDI-TOF MS analysis, the precipitates were dissolved in N,N-dimethylformamide (DMF), and the reaction solutions were used as such. A volume of 2 μL of DMF sample or reaction solution was mixed in a 1:3 ratio (v/v) with a solution of 2,5-dihydroxybenzoic acid, DHB (purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany)). DHB solution was prepared by dissolving 1.5 mg into 150 μL of water containing 50% acetonitrile (ACN) and 0.1% trifluoro acetic acid (TFA) from Sigma-Aldrich (Allentown, USA). For the MS measurement, 2 μL of the sample-matrix mixture was dried onto the MALDI target plate at room temperature. The positive ion mass spectra were acquired from the dried sample spots in reflector mode. The number of scans was typically 2000. Peptide standard solution purchased from Bruker (Bremen, Germany) was used for the molecular mass calibration.

Chemo-Enzymatic Modification of NFC

Laccase-catalyzed chemo-enzymatic modification reactions of NFC were carried out in 1% (w/v) water suspension at 45 °C (pH 4.5) in open flasks under constant stirring to ensure availability of oxygen for ThL. Enzymatic treatment was started by reacting NFC for 30 min. with ThL before adding DOGA to the reaction suspension. After 90 min. total reaction time with NFC, ThL, and DOGA, the enzymatic reaction was terminated by boiling samples for 10 min. in a water bath. ThL and DOGA dosages used were 500 nkat and 0.12 mmol per gram of dry solid NFC, respectively. The amount of DOGA corresponded approximately 4% (w/w) of the dry solid content of the NFC. Reference treatments were carried out similarly but without addition of ThL and/or modifying chemical. Samples were not washed after enzymatic treatment due to poor solubility of DOGA in water. Organic solvents were avoided as they could have influenced the physico-chemical properties of NFC. Additionally, some nanoscale material would have been lost during washing process.

Surface Properties of Chemo-Enzymatically Modified NFC

Free-standing films and coatings were prepared from the native and modified NFC to test hydrophilicity and topography of the surfaces. Free-standing films were prepared by filtration. Membrane filter Durapore® HVLP04700 (0.45 μm; 47 mm diameter) from Millipore (Ireland) was placed on a filtered glass funnel where NFC at 0.5% (w/v) concentration was decanted. Unbound water was removed by suction through the membrane filter. Final dehydration was conducted at 50% relative humidity at room temperature. To prepare coatings for the characterization, NFC was diluted to 0.8% (w/v) concentration and filter paper Whatman 3 (Whatman International Ltd, England) was hand-coated with a rod as a 200 μm wet layer. Initial dehydration of coatings was conducted at 80 °C for 15 min., and final drying at 50% relative humidity at room temperature.

Contact angle (CA) measurements of NFC films and coatings were performed with a Modular CAM-200 system, (KSV Instruments, Finland) using distilled water as the solvent. The measurement was continued at one-second intervals for 5 min. The CA value of the sample was calculated as the average of the first 3 time points. Triplicate measurements were carried out for each sample.

Surface topography and roughness (R_a) of NFC films were analyzed by optical profiler Veeco Wyko NT9100 (Veeco Metrology Group, Tucson, Arizona, USA). For the determination of R_a, test points varying from 5 to 10 with areas of 236 x 314 μm were used in the analysis.

Tension Strength of Chemo-Enzymatically Modified NFC

Free-standing films for characterization of mechanical properties were prepared by solvent casting. A 9 cm diameter Petri-dish was used to dry 50 g of 1% (w/w) NFC at 45 °C. Thickness of the obtained films was about 80 μm. Test specimens with 40 mm length were cut with a film cutter using blade distance of 5.3 mm. The specimens were conditioned at 23 °C in 50% relative humidity for three to five days, and the thickness was measured from each specimen with a Mitutoyo 293-676 Quickmike Digimatic Micrometer with 0.001 mm resolution. Tensile properties of at least five specimens were measured with an Instron 4204 testing machine with a test speed of 1 mm min.$^{-1}$.

RESULTS AND DISCUSSION

Characterization of NFC Materials

The amount of nanoscale material and rheological properties were determined to characterize the NFCs. The portion of nanoscale material, *i.e.* material that stayed in the supernatant after extensive centrifugation of fluidized NFC, was 40% and 33% of the dry content of the unbleached and bleached NFC, respectively. The rheological measurements showed that both samples exhibited gel-like character (G' >> G'') at the frequency used in the measurements (Table 2). The bleached sample had somewhat higher storage (G') and loss modulus (G'') values in the linear viscoelastic region than the unbleached one, but the bleached material was slightly less elastic based on its higher phase angle value. The samples were very similar in terms of the critical stress needed to break the gel network. Slight differences in the amount of nanoscale material and rheological properties can be explained from different defibrillation properties and different diameter of resulting fibrils from unbleached and bleached pulps (Spence *et al.* 2010a,b), as well as differences in the fibrillation process.

Table 2. Viscoelastic Properties of Unbleached and Bleached NFC Gels as Determined by Oscillatory Stress Sweep Measurements *

	G' (Pa)	G'' (Pa)	Phase angle (°)	Critical Stress (Pa)
Unbleached				
Average	321.3	40.0	7.2	46.0
St dev	7.6	1.0	0.1	6.1
Bleached				
Average	513.3	81.0	9.0	50.0
St dev	3.8	1.0	0.1	0.0

*Storage Modulus (G'), Loss Modulus (G'') and Phase Angle were determined from the linear viscoelastic region.

According to the chemical and rheological analyses, as well as the degree of fibrillation, unbleached and bleached NFCs were rather similar except for the lignin content. According to Spence *et al.* (2010b), the lignin content does not have a major effect on the morphology of films prepared from fibrillated pulp. Thus, it was assumed that unbleached and bleached NFCs were nearly identical to one another in terms of their suitability to be used as test materials to evaluate the effect of lignin content on surface properties of films and coatings prepared after chemo-enzymatic treatment.

Reactivity of Laccase with NFC, Model Compounds, and Functional Chemicals

The reactivity of NFC and phenolic derivatizers with ThL was verified by oxygen consumption measurements prior to actual coupling reactions. Oxygen consumption curves for the two different types of NFCs, the phenolic derivatizers (PROGA and DOGA), and the lignin model compound (EROL) are shown in Fig. 1. The initial rate of oxygen consumption with DOGA and PROGA was on same level. Oxidation of DOGA levelled off faster as compared to that of PROGA. Initial concentration of dissolved oxygen was 5 mg L^{-1} in the DOGA solution at 45 ºC, and 8 mg L^{-1} in the PROGA solution at room temperature. Oxidation of DOGA might be restricted by the poor

solubility of DOGA in water or oxygen availability later in the reaction. The initial rate of oxidation of EROL was *ca.* 40% slower than that of PROGA. One reasonable explanation of this observation was the oxidation potentials of PROGA and EROL in reaction conditions applied (pH 4.5) were significantly different. The oxidation potential of gallates is around 0.4 V (Gunckel *et al.* 1998; Díaz González *et al.* 2009), and the gallates can undergo several subsequent oxidations after formation of the first oxidation product. For guaiacylic derivatives, such as EROL, the corresponding oxidation potential values are significantly higher, around 0.6 to 0.7 V (Díaz González *et al.* 2009). The presence of 10% acetone (v/v) in the EROL sample was assumed to have a very minor effect on the ThL reaction, as ThL lost only 10% of its activity in the 10% acetone solution. In general, different dilute organic solvents have been found to have only a minor effect on the stability and the activity of laccases (Mattinen *et al.* 2011) After 60 min. reaction time, 27% of DOGA, 67% of PROGA, and 50% of EROL was oxidized.

In addition to the soluble compounds, measurement of dissolved oxygen can be used to study the reactivity of different lignocellulosic fibre materials; however, there is not necessarily a direct correlation between the amount of oxygen consumed and radicals formed with all fibre types. Linear correlations have been reported with beech wood fibres (Felby *et al.* 1997), but there are also some examples reported where the formation of radicals have been detected in spite of there being no observed oxygen consumption by thermomechanical and chemi-thermomechanical pulp fibres (Suurnäkki *et al.* 2010; Grönqvist *et al.* 2003). As expected, ThL could not oxidize the fully bleached NFC, whereas the oxidation of unbleached NFC containing residual lignin was clear (Fig. 2). Thus, the activation of fully bleached NFC was not possible through ThL action due to its very low lignin content.

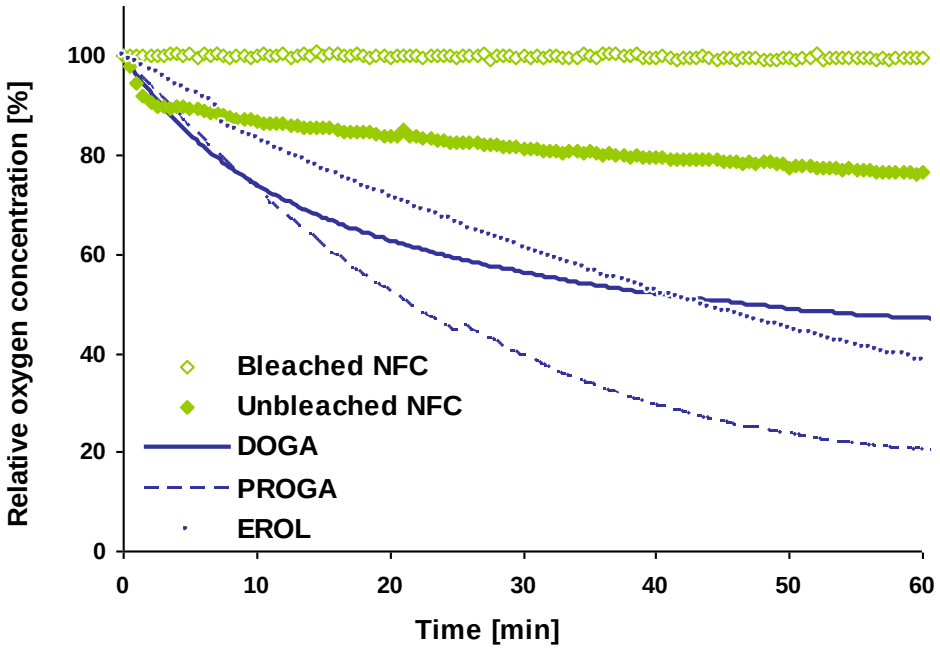


Fig. 2. Relative concentration of dissolved oxygen as a function of time when bleached, and unbleached NFC, DOGA, PROGA, and EROL were treated by ThL. Measurements were carried out at pH 4.5 at room temperature, except with DOGA, which was carried out at 45 °C.

Enzymatic Polymerization and Coupling of Model Compounds and Functional Chemicals

Reaction kinetics of ThL catalyzed oxidation of PROGA and EROL

Formation of polymerized products of PROGA and EROL as a function of time during the ThL catalyzed oxidation was studied by HPLC. PROGA and EROL were reacted with ThL, both individually and concurrently. Polymerization reactions of both compounds were fast, and substantial proportion of both compounds were polymerized after two minutes reaction time.

Polymers of EROL and PROGA were formed after certain intermediate products were generated. PROGA was fully reacted after 120 min. reaction time, resulting in a very wide scale of different polymerization products. After 30 min. reaction time, the EROL was completely reacted, with subsequent reactions of different intermediate polymerization products occurring to form other polymeric products. The presence of both components, EROL and PROGA, in the reaction solution substantially affected their reaction velocities.

PROGA and EROL seemed to compete to react with the laccase. The presence of PROGA delayed the formation of EROL reaction products. Polymerization products of EROL were formed only after the PROGA was consumed by its polymerization reactions.

Enzymatic polymerization of DOGA, PROGA and EROL by ThL

The long hydrophobic hydrocarbon chain of DOGA, as shown in Fig. 1, decreased its solubility in water. However, it was possible to prepare a dispersion of DOGA (Buchert *et al.* 2007). When the dispersion of DOGA was diluted, it instantaneously aggregated. PROGA was soluble in water until its concentration was 3.5 g L^{-1}. Apparently, changes took place with DOGA and PROGA solutions when they were treated with ThL.

The DOGA solution was initially white and opaque when the ThL was added. After the ThL addition, the reaction mixture became gingery in color and became clear. Also, in the case of PROGA, a ginger-colored solution was obtained during the ThL catalysed oxidation, and a precipitate was formed at high substrate concentrations. Polymerization of DOGA as a result of oxidative reactions with ThL was first verified by SEC (data not shown). To obtain more detailed structural information of the reaction products, the formed polymers of DOGA, PROGA and EROL by ThL were analysed by MALDI-TOF MS (Fig. 3).

Detected, as well as predicted m/z values of the polymerize-tion products containing 3 to 6 DOGA, PROGA or EROL units are shown in Table 3. In the polymer series of EROL, there was a detected mass difference of 124.0524 g mol^{-1} (symbol E*), which probably was a degradation product of EROL. Masses of homopolymers were calculated according to the equation $[n\mathrm{MM} - 2H(n\text{-}1) + \mathrm{Na}^+]$, where n was the number of monomers, MM was the monoisotopic molecular mass of DOGA, PROGA or EROL, and H was the monoisotopic molecular mass of hydrogen. Corresponding polymerization reactions of simple phenolic compounds, such as ferulic acid, by ThL has also been verified by Mattinen *et al.* (2005). Oxidative polymerization of phenols and polyphenols by oxidative enzymes, like horseradish peroxidase and laccase, has been recently reviewed by Kobayashi and Makino (2009).

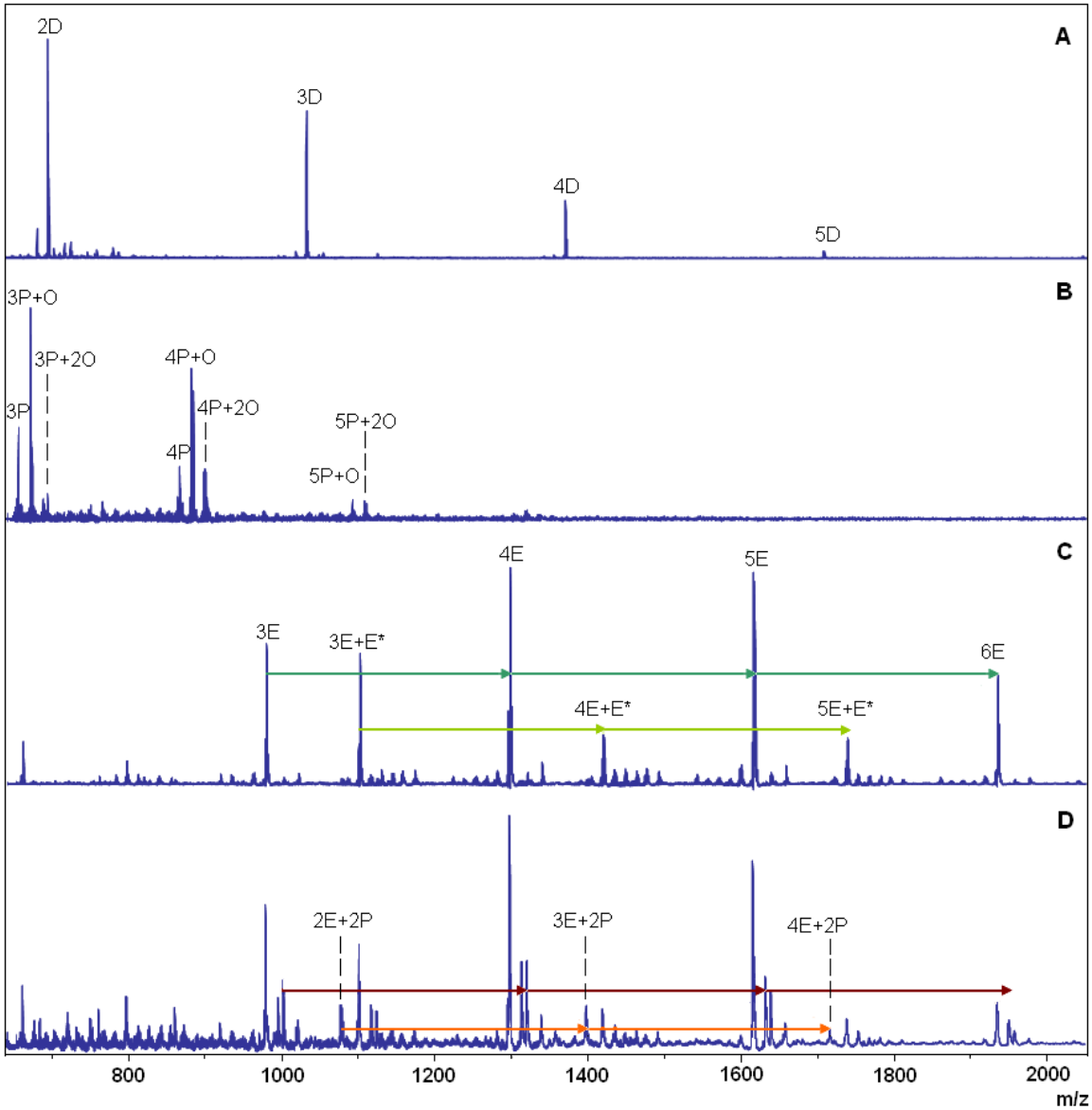


Fig. 3. MALDI-TOF MS of polymerization products of (A) DOGA (abbrev. D), (B) PROGA (abbrev. P), (C) EROL (abbrev. E, where E* means degradation product of E), and (D) mixture of homo and hetero polymers of PROGA and EROL. The abbreviation O refers to oxygen. Arrows indicates mass difference of 318 Da. Polymer series of PROGA was detected from the solution, not from the precipitate, as with the other series.

Enzymatic coupling of PROGA and EROL

Covalent binding of the aromatic ring of gallate to the aromatic ring of a lignin model compound was examined by MALDI-TOF MS to demonstrate the possibility of covalent functionalization of residual lignin present in NFC. On the basis of oxygen consumption measurement, the initial oxidation velocity of DOGA and PROGA by ThL was nearly identical. However, due to its higher water solubility, PROGA was selected as a model compound to demonstrate the covalent chemical bond formation between EROL and gallate molecule. The higher reactivity of PROGA over EROL on the basis of oxygen consumption measurements and HPLC analyses was taken into account in dosages of PROGA and EROL during coupling reaction for MALDI-TOF MS analyses.

Table 3. Detected and Predicted Masses of Homopolymers of DOGA, PROGA, and EROL after Treatments with ThL.**

Compound	Predicted m/z (Da)	Detected m/z (Da)	Δ m/z (Da)
Part A in Fig 3.			
$(DOGA)_2Na^+$	697.39	697.31	0.08
$(DOGA)_3Na^+$	1033.59	1033.76	-0.18
$(DOGA)_4Na^+$	1369.78	1370.21	-0.43
$(DOGA)_5Na^+$	1705.97	1706.65	-0.68
$(DOGA)_6Na^+$	2042.17	2043.06	-0.90
Part B in Fig 3.			
$(PROGA)_3\,Na^+$	655.16	654.98	0.19
$(PROGA)_4\,Na^+$	865.22	865.13	0.09
$(PROGA)_3\,O\,Na^+$	671.16	670.99	0.17
$(PROGA)_4\,O\,Na^+$	881.21	881.14	0.07
$(PROGA)_5\,O\,Na^+$	1091.26	1091.30	-0.04
$(PROGA)_3\,2O\,Na^+$	687.15	686.92	0.23
$(PROGA)_4\,2O\,Na^+$	897.21	897.14	0.06
$(PROGA)_5\,2O\,Na^+$	1107.26	1107.31	-0.05
Part C in Fig 3.			
$(EROL)_3\,Na^+$	979.34	979.21	0.13
$(EROL)_4\,Na^+$	1297.45	1297.44	0.01
$(EROL)_5\,Na^+$	1615.56	1615.70	-0.14
$(EROL)_6\,Na^+$	1933.67	1933.97	-0.30
$(EROL^*)(EROL)_3\,Na^+$	1101.37	1101.29	0.09
$(EROL^*)(EROL)_4\,Na^+$	1419.48	1419.55	-0.07
$(EROL^*)(EROL)_5\,Na^+$	1737.59	1737.80	-0.21
Part D in Fig 3.			
$(EROL)_4\,O\,Na^+$	1313.44	1313.39	0.05
$(EROL)_5\,O\,Na^+$	1631.55	1631.64	-0.09
$(EROL)_6\,O\,Na^+$	1949.66	1949.87	-0.21
$(PROGA)_2(EROL)_2\,Na^+$	1081.33	1080.35	0.98
$(PROGA)_2(EROL)_3\,Na^+$	1399.44	1398.45	0.99
$(PROGA)_2(EROL)_4\,Na^+$	1717.55	1716.70	0.85
EROL* refers to degradation products of EROL.			

** Also some of the identified heteropolymers of PROGA and EROL are listed.

Determination of suitable substrate concentrations for enzymatic coupling reactions was essential to be able to shift the reaction equilibrium toward the wanted reaction products, *i.e*. heteropolymers. According to HPLC analysis, the reactions of PROGA by ThL were in competition with the reactions of EROL by ThL, and thus, a lower concentration of PROGA compared to concentration of EROL in coupling reactions was used to avoid its unilateral homopolymerization. When the PROGA and EROL mixture was treated by the high redox ThL, in addition to homopolymers, some novel polymer series were detected, which suggested the formation of PROGA-EROL heteropolymers as shown in Fig. 3D. Masses of the heteropolymers were calculated according to equation $[n_1MM_1 + n_2MM_2 - 2H(n_1 + n_2 -1) + Na^+]$, where n was the number of monomers, MM was the monoisotopic molecular mass of PROGA or EROL, and H was the monoisotopic molecular mass of hydrogen.

It has been shown that, in addition to polymerization reactions of phenolic compounds by laccase *via* coupling of different radicals (Mattinen *et al.* 2005; 2008), different types of rearrangements leading to C_α oxidation or cleavage of alkyl–aryl bonds, as well as other partial degradation products, takes place (Xu 2005). The oxidation of PROGA proceeded either by radical coupling or by two-electron oxidation, leading to irreversible formation of unstable ortho-quinones. The addition of nucleophiles, either in form of another molecule of PROGA, or water, to the C_2- or C_6-position of the aromatic ring yields several possible reaction products (Kubo *et al.* 2003; 2010), which are also susceptible to further oxidation by laccase. Combination of these reactions led to the formation of extremely complex heterocompound and heteropolymer structures, in addition to the formation of homopolymers.

It has been also speculated that different dehydropolymers may form due to the laser irradiation of the polymerized products during the MALDI-TOF MS analysis (Gao *et al.* 2003), in addition to laccase-catalysed reactions (Mattinen *et al.* 2011). Thus, the detailed structural characterisation of the PROGA-EROL heteropolymers was difficult to assess; other soft ionization mass spectrometric techniques, as electrospray ionization mass spectrometry (ESI-MS) combined with the chromatographic separation, would be necessary for structural elucidation of the polymerised hetero-oligomers (Kudanga *et al.* 2010b). Hence, even though all exact structures of hetero products of EROL and PROGA could not be specifically characterized, the masses of the identified novel peaks are reported in Table 3.

Surface Properties of Chemo-Enzymatically Modified NFC

Chemo-enzymatic modification of NFC properties by ThL-catalyzed coupling of DOGA was evaluated from two different NFC film surfaces: free-standing film and paper coating. Film formation of NFC is based on hydrogen bonding between fibrils during drying. The time allowed for fibrils to arrange to form the bonds greatly affects the film properties. Thus, to characterize the modifications, two kinds of film surfaces of NFC were prepared by altering the drying time. Free-standing films were dried slowly at 50% relative humidity at room temperature, whilst coatings were rapidly dried at 80°C. Characteristics of chemo-enzymatically modified NFC were examined by contact angle measurements. Contact angle was assumed to increase due to hydrophobic nature of the grafted derivatizing agent, DOGA (Fig. 1), used in the modification. Despite the relative large standard deviation of contact angle results within one sample and between separate samples, some repeated trends were observed. The trends were different in free-standing films and paper coatings, as shown in Fig. 4. Since the samples were unwashed, the amount of DOGA was the same in samples with and without ThL treatment. Thus, the differences in contact angle between pure DOGA and ThL+DOGA-treated NFC samples could only be derived from reactions originating from laccase catalyzation.

Free-standing films

Laccase-catalyzed DOGA modification of NFC had only a minor effect on the contact angle of free-standing NFC films (Fig. 4A). Furthermore, no clear difference in the effect of ThL+DOGA modification on contact angle as compared with the untreated reference was observed in any case. These results indicated that oxidative coupling of DOGA to NFC lignin was not a significant factor affecting the hydrophilic properties of the self-standing NFC films surfaces.

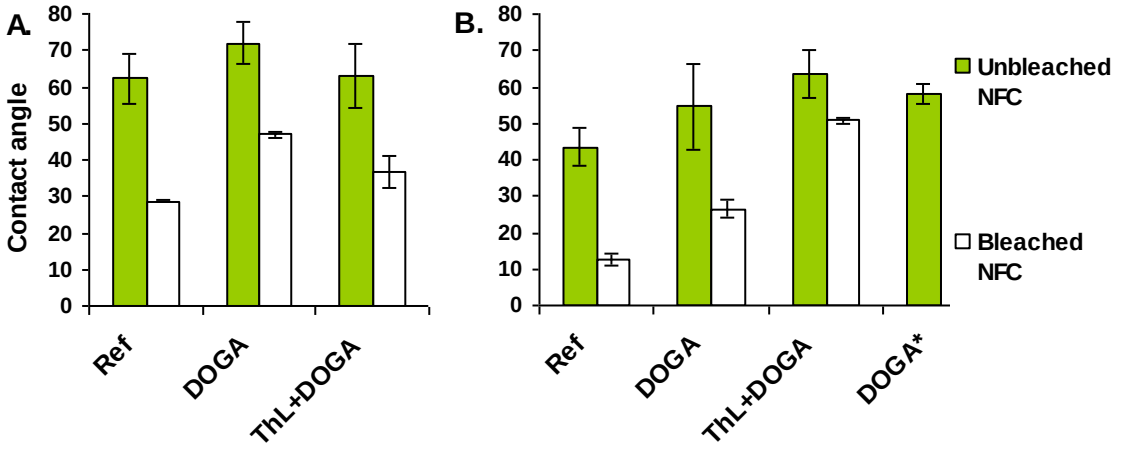


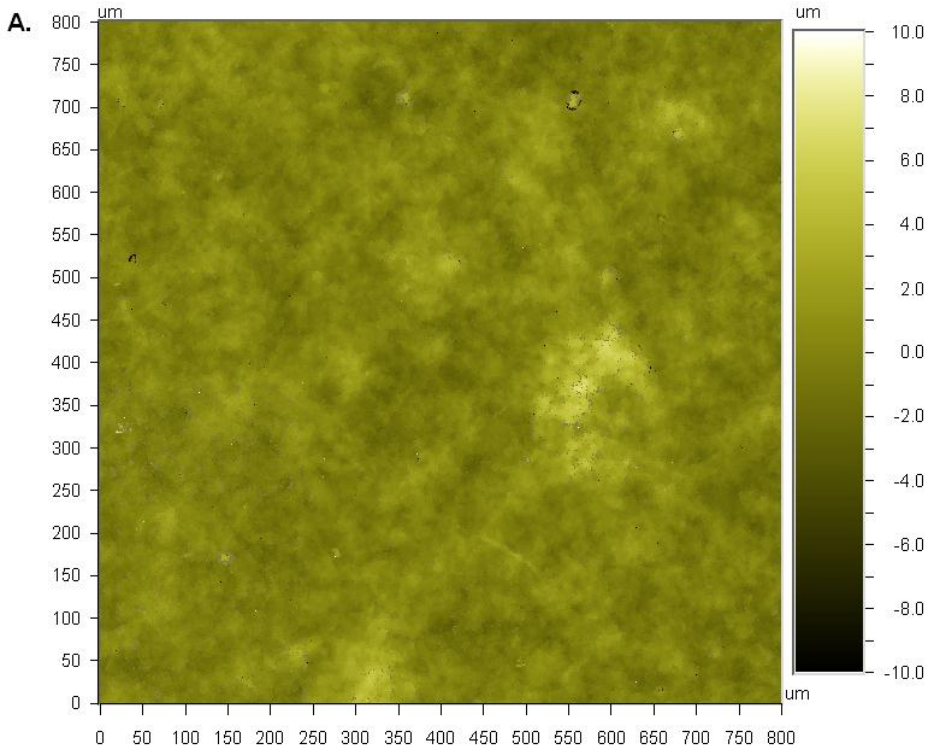
Fig. 4. Contact angles of NFC (A) free-standing films and (B) paper coatings. Values of unbleached, lignin containing NFC were averages of three experiments in free-standing films and of six experiments in coatings. Values of bleached NFC were from one experiment. Ref = treatment of NFC without derivatizing agent or enzyme, DOGA = dispersion of DOGA was mixed with NFC in the treatment, ThL+DOGA = chemo-enzymatic treatment with DOGA using laccase activation DOGA* = enzymatically pre-polymerized DOGA was mixed with NFC.

Films prepared from bleached or unbleached NFC, together with native DOGA, gave higher contact angle values than the reference films or films prepared from ThL + DOGA-treated NFCs (Fig. 4A). The reason for the different effects of native and laccase-polymerized DOGA for film surface contact angle could be derived from their different behavior in water suspensions. In water, non-polymerized DOGA aggregated and accumulated, whilst polymerized DOGA remained well-dispersed, as stated earlier in reference to the enzymatic polymerization studies of the model compounds.

To study if the same behavior took place during slow drying of free-standing films from water based systems, topography and roughness of the films was measured. The aggregates of non-polymerized DOGA on film surface can be seen in topographic images of free-standing films (Fig. 5).

Surface topography and surface roughness of sample of ThL + laccase-treated NFC was similar to those of reference sample, whereas the native DOGA-containing sample had clearly higher surface roughness as compared to reference sample (Table 4). The increase in surface roughness, together with the increased contact angle, suggested that native DOGA had accumulated at the NFC film surface. This accumulation of DOGA could have been promoted by hydrogen bond formation between the hydroxyl groups of cellulose fibrils, and consequently the inter-fibrillar bonding and tight network formation of the nanocellulose fibrils during drying.

Unlike native DOGA, laccase-polymerized DOGA had no tendency for aggregation or accumulation in water; in addition, the accumulation of large DOGA polymers at the NFC matrix surface would be difficult due to the fibril network. Also, if the DOGA was coupled to the lignin due to laccase action, that would have prevented its accumulation onto the surface.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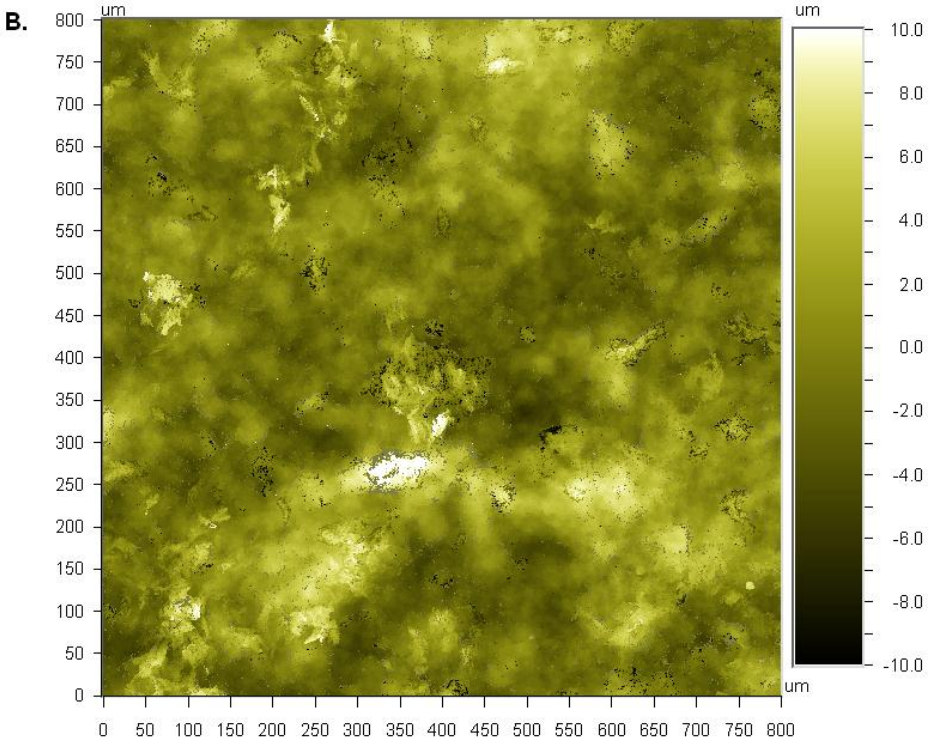
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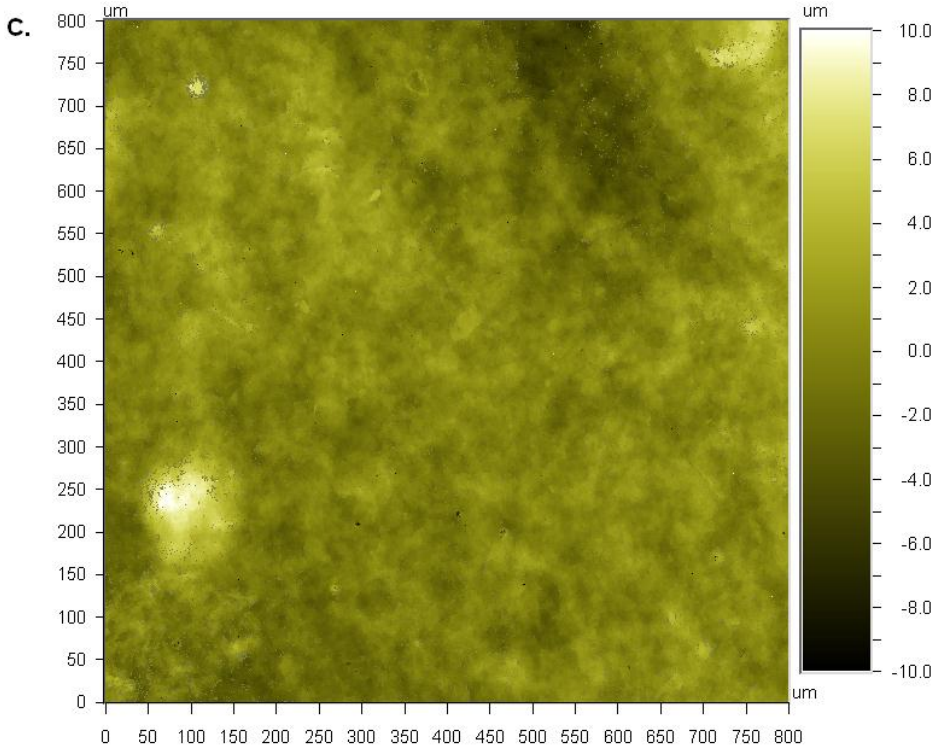

Fig. 5. Surface topographic images of dried NFC free-standing films. A: Reference NFC film; B: NFC with DOGA; and C: NFC with DOGA after laccase catalyzed reactions

Table 4. Average Surface Roughness of Free-standing Films made from Unbleached NFC

Sample	Roughness (μm)	Standard deviation (μm)
Reference	0.8	0.2
DOGA	2.0	0.3
ThL + DOGA	1.0	0.3

Filter paper coating

In the paper coatings, clearly higher contact angle levels were measured from the samples coated with ThL + DOGA-treated NFC (Fig. 4B). In the case of unbleached lignin containing NFC, the contact angle increased by 44% as compared with the reference sample due to ThL + DOGA treatment. The addition of native DOGA to NFC also increased the contact angle of the final coating, although less than that of laccase-DOGA treatment. As in the case of free-standing films, covalent bonding of the DOGA to the residual lignin present in unbleached NFC could not be the main factor affecting the contact angle, since the laccase-DOGA treatment also increased the contact angle of bleached NFC coatings (Fig. 4B). To clarify the role of laccase-polymerized but unbound DOGA to the contact angle of NFC coatings, DOGA pre-polymerized with laccase was blended with the native unbleached NFC, whereafter this NFC sample was used to coat the paper and the coating was analysed for contact angle. The contact angle value of the coating of NFC-blended with the pre-polymerised DOGA was approximately on the

same level to that of the NFC-treated with laccase and DOGA (Fig. 4B). As stated earlier, molecular mass of DOGA was increased (Table 3) and its dispersion in water was improved due to laccase-catalysed polymerization. Thus, it could have formed a uniformly spread network on NFC surface during coating. That network could have increased the water contact angle of the coated filter via hydrophobic hydrocarbon chains of DOGA and by sealing filter pores. In native DOGA-containing samples, un-polymerized DOGA could have formed aggregates, which would be randomly scattered on the surface. This could also explain the larger standard deviation of the contact angle for the native DOGA containing NFC sample as compared to standard deviations of the reference and laccase-DOGA treated NFC samples.

Mechanical Properties of Modified Unbleached NFC

The action of laccase with lignin-rich fiber materials is known to enhance the internal bonding of fibers and hence improve the mechanical properties. Internal bonding of fibers is conducted from covalent bonding via radicals formed in lignin and from improved inter-facial compatibility of fibers (Felby *et al.* 2002; 2004). Tensile tests of free-standing films of chemo-enzymatically treated unbleached NFC were performed to examine effect of laccase and chemo-enzymatic treatment on mechanical properties of unbleached NFC. Comparison to bleached NFC was also made, and the results are shown in Table 5. Bleached NFC had higher modulus and strength than unbleached NFC due to better arrangement of fibrils through physical interactions during drying. When residual lignin was present, it prevented the formation of repetitive hydrogen bonds between the fibrils. Toughness, measured as maximum elongation, was slightly higher when lignin was present. By laccase treatment of the unbleached NFC, a cross-linked product with increased stiffness and strength was gained. Lignin, which is naturally strongly bound to the cellulose chains by physical bonds, was proposed to be cross-linked due to laccase-catalyzed oxidation, and possibly formed bridges through the DOGA molecules.

The significance of both reagents, laccase and DOGA, was evaluated by measuring the mechanical properties of free-standing films prepared without one component. When laccase was not added, the presence of only DOGA caused a decrease in mechanical properties. In contrast, addition of only laccase increased the stiffness and strength significantly, and similar mechanical properties were gained as with the chemo-enzymatic treatment. These results strengthen the observation that DOGA was very well integrated into the NFC matrix due to the action of laccase.

Table 5. Young's Modulus (E), Tensile Strength (σ), and Elongation at Break (ε) of Free-standing Films *

Sample	E (MPa)	σ (MPa)	ε (%)
Bleached NFC	7440 (530)	150 (8)	10.2 (1.0)
Unbleached NFC	6540 (690)	139 (5)	12.0 (1.3)
Unbleached NFC + ThL	7200 (720)	145 (8)	9.7 (1.2)
Unbleached NFC + DOGA	6230 (540)	116 (9)	9.4 (3.2)
Unbleached NFC + ThL + DOGA	7660 (740)	135 (9)	9.4 (1.3)

* Standard deviation is shown in parenthesis.

CONCLUSIONS

1. Polymerization of DOGA and PROGA by ThL was proven by the MALDI-TOF MS analysis. Polymerization affected the dispersion properties of DOGA in water solution, and hence its behavior in the NFC matrix.
2. MALDI-TOF MS analyses with lignin model compounds EROL and PROGA suggested that covalent coupling *via* phenolics in lignin and gallate took place.
3. Covalent bonding between DOGA and NFC was not the main factor affecting the surface properties of NFC in free-standing films or paper coatings. ThL-catalysed polymerization of DOGA affected its dispersion properties in water, which affected its behavior in water-based NFC matrix. It was observed that addition of pre-polymerized DOGA to NFC influenced the surface properties of NFC significantly.
4. By laccase treatment of the unbleached NFC, a cross-linked product with increased stiffness and strength was obtained with and without DOGA. If the DOGA was mixed with the unbleached NFC without laccase, the mechanical properties of free-standing film deteriorated.
5. Sample preparation method affected the detection of NFC modification. By ThL-DOGA treatment, less hydrophilic surface properties were detected in the coatings; however, in the free-standing films, no modifications to the surface properties were detected.

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