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*Published in:*
Enzyme and Microbial Technology

*DOI:*
10.1016/j.enzmictec.2018.01.005

*Published: 01/04/2018*

*Document Version*
Publisher's PDF, also known as Version of record

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*Please cite the original version:*
https://doi.org/10.1016/j.enzmictec.2018.01.005
Enzymatically and chemically oxidized lignin nanoparticles for biomaterial applications

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\begin{abstract}
Cross-linked and decolorized lignin nanoparticles (LNPs) were prepared enzymatically and chemically from softwood Kraft lignin. Colloidal lignin particles (CLPs, ca. 200 nm) in a non-malodorous aqueous dispersion could be dried and redispersed in tetrahydrofuran (THF) or in water retaining their stability i.e. spherical shape and size. Two fungal laccases, \textit{Trametes hirsuta} (ThL) and \textit{Melanocarpus albomyces} (MaL) were used in the cross-linking reactions. Reactivity of ThL and MaL on Lignoboost™ lignin and LNPs was confirmed by high performance size exclusion chromatography (HPSEC) and oxygen consumption measurements with simultaneous detection of red-brown color due to the formation of quinones. Zeta potential measurements verified oxidation of LNPs via formation of surface-oriented carboxylic acid groups. Dynamic light scattering (DLS) revealed minor changes in the particle size distributions of LNPs after laccase catalyzed radicalization, indicating preferably covalent intraparticular cross-linking over polymerization. Changes in the surface morphology of laccase treated LNPs were imaged by atomic force (AFM) and transmission emission (TEM) microscopy. Furthermore, decolorization of LNPs without degradation was obtained using ultrasonication with H$_2$O$_2$ in alkaline reaction conditions. The research results have high impact for the utilization of Kraft lignin as nanosized colloidal particles in advanced bionanomaterial applications in medicine, foods and cosmetics including different sectors from chemical industry.
\end{abstract}

\section{1. Introduction}
Lignin, one of the main polymers of lignocellulosic biomass, co-stream from biorefinery and paper industry, is a sustainable and renewable resource \cite{1}. It is a self-associating aromatic biopolymer providing strength and rigidity for plant stems, also controlling the fluid flow. Due to its stabilizing, thermal, antioxidative, and antimicrobial properties, lignin is a promising raw material for different biomaterials. High technology applications include UV-protective agents for cosmetics and in medicine materials for drug delivery and tissue repairing \cite{3,2}. Due to the complex structure, much of the lignin research during the past decades has been focused on the technology and analytical method development for the isolation and characterization of lignin. The improvement of the thermal and mechanical properties of lignin \cite{4}, including the discovery of catalytic pathways to produce renewable alternatives to petroleum-based chemicals, polymers, fibers, and fuels have been central in the research field \cite{5}. In addition to its brown color and unpleasant odor \cite{6}, the low solubility and poor miscibility of lignin in organic solvents and various formulations have been major challenges for the development of novel lignin-based biomaterials \cite{4}. In the advanced applications, technical lignin is often utilized as nanoparticles to overcome these limitations \cite{2}.

Green nano- and biotechnologies are rapidly growing research fields. The use of plant polymers as nanosized additives in materials is increasing rapidly \cite{7}. Colloidal lignin particles (CLPs) could also be interesting nanosized components for many biomaterial applications due to their smooth surface structure and stability in physiological conditions \cite{7}. However, these particles have gained considerably less attention than nanocellulose.

Several methods have been developed for the preparation of LNPs...
[8–17]. In our previous study, it was shown that spherical LNPs could be produced from Kraft lignin without any chemical modification by dissolving lignin in tetrahydrofuran (THF) and subsequently changing the polarity of the solvent by exchange to water via dialysis [7]. The current estimation for the costs to prepare CLPs is below one €/kg on dry basis. Besides their potential use for advanced applications [3,18,19], LNPs could be used to stabilize Pickering emulsions and foams for household products and building materials [20–22]. Other technical applications include organic fillers for nanocomposites, packing and natural rubber to be used as partial replacement of carbon black [23,24]. LNPs modified with silver ions have lower environmental impact than metallic silver nanoparticles [25] to be used e.g. in textiles as antimicrobial agents for fibre modification.

Enzymatic cross-linking with laccases (oxidoreductases, EC 1.10.3.2) [26–29] could be a potential method to reinforce spherical morphology of LNPs against dissolution in organic solvents. Such a property is important for several industrial applications e.g. for adhesives and coatings where LNP could be exploited as surfactants or binders [2]. The oxidation of phenolic groups of lignin via radical formation with and without mediators following polymerization and cross-linking reactions has been recently reviewed [30,31]. Covalent cross-links can also be formed when lignin is heated at high temperatures or reacted with epichlorohydrin (1-chloro-2,3-epoxypropane) [20] as well as using metal–biogenic sol–gel reactions [32–35]. Controlled radical polymerization based on the reversible addition-fragmentation chain transfer (RAFT) mechanism [36] has been successfully used to tailor LNP surfaces with polyacrylamide.

In the present contribution, aqueous LNP dispersions were treated with high Trametes hirsuta (ThL) and low Melanocarpus albomyces (MaL) redox potential laccases to improve the colloidal stability of the particles in the model organic solvent (THF) via surface and intraparticle cross-linking. Furthermore, it was shown that ultrasonication in alkaline conditions with H2O2 could be used to decolorize LNP dispersions at low temperature.

2. Materials and methods

2.1. Enzymes

Low redox potential MaL (0.47 V), pH optimum determined against guaiacol, was overproduced in Trichoderma reesei and purified as described previously [37,38]. High redox potential ThL (0.78 V), pH optimum measured against the same substrate [39], was produced in its native host and purified as described Rittstieg [40]. The activities of both laccase preparations were analyzed using 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfonate (ABTS) as substrate [39]. The oxidation of ABTS by laccases at pH 4.5 and in 25 mM Na-succinate buffer was analyzed by Perkin Elmer Lambda 45 spectrophotometer (MA, USA) at 436 nm (ε = 29,300 M⁻¹ cm⁻¹). For ThL preparation, the specific activity was 1500 nkat mg⁻¹ and for MaL it was 250 nkat mg⁻¹.

2.2. Preparation of LNP dispersions

Softwood LignoBoost™ Kraft lignin [6] from Domtar plant (NC, USA) was used to prepare aqueous LNP dispersions as described Lievonen et al. [7]. The chemical structure of the lignin was also characterized previously [7]. The concentration of the colloidal lignin particles (ca. 200 nm) in aqueous stock dispersion was 4 mg ml⁻¹ and pH 4.3. For the enzymatic cross-linking studies, preparative ultra-centrifuge (Optima™ L Series, rotor type 70 Ti, Beckman Coulter, Sweden) was used to remove minor amount unstable colloidal particles. Centrifugation speed 2000 rpm (G-force 1000) for 30 min following syringe filtration (0.45 μm) clarified the LNP dispersion. The pH for the laccases was adjusted with 0.1 mM HCl and 0.1 mM NaOH. For the chemical decolorization of LNPs the stock dispersion was centrifuged using 5000 rpm (G-force 1800) for 20 min.

2.3. Reactivity of laccases

First, the reactivity of laccases on non-particulate LignoBoost™ lignin was examined by aqueous HPSEC. For the analyses, powdered Kraft lignin was dissolved in alkalii (0.1 mg ml⁻¹ and 0.5 mg ml⁻¹) as described by Moya et al. [27]. Laccase activity was 500 nkat g⁻¹ and treatment time 20 h at pH 6 to maintain polymerized reaction products in the solution. Acid precipitation (pH 2) was used to separate enzymatically polymerized lignin, which was washed with water and ultracentrifuged (Optima™ L Series, rotor type 70 Ti, Beckman Coulter, Sweden) until the pH of the supernatant was 5.5 and finally dried at 80 °C. For the chromatographic separations, all the enzymatically polymerized lignins and molecular weight standards (194 Da–0.1 kDa) were dissolved in 0.1 M NaOH. Weight-average molar mass (Mw) of the samples was analyzed using Agilent 1260 Infinity high performance size exclusion chromatography (HPSEC) system equipped with a UV detector (USA) as described recently [41].

Then the reactivity of laccases on LNPs was followed by oxygen consumption measurement. The enzyme activities were 500 nkat g⁻¹ and 1000 nkat g⁻¹ and substrate concentration 0.1 mg ml⁻¹ and 1.0 mg ml⁻¹ at pH 4.5 for ThL and pH 7.6 for MaL treatments. Additionally, the reactivity of the laccases on LNPs was compared at equal pH 5.0 using the enzyme dosage 1000 nkat g⁻¹ and 1.0 mg ml⁻¹ substrate concentration. LNP dispersions were stabilized (30 min) in open reaction vessels to ensure maximal dissolution of oxygen at ambient temperature for the enzymatic oxidation. After laccase addition, consumption of dissolved oxygen was followed (4 h) using a fibre-optic oxygen meter (OXY-10, PreSens, Germany). The measurements were performed under constant mixing in glass vessels (1.8 ml), filled and sealed to avoid the entry of oxygen into the reaction mixtures during the experiments. Finally, the reaction vessels were left open (24 h) under constant magnetic stirring to complete enzymatic reactions. Furthermore, reactivity of MaL and ThL on small molecule lignin model compound, guaiacol (Merck, Germany) was compared at pH 5.0 using enzyme dosage 0.15 nkat g⁻¹ and substrate concentration 0.1 mg ml⁻¹.

The reactivity of laccases on LNPs was also verified visually. After overnight incubation, intense red-brown color was expected to be formed in dilute LNP dispersions due to formation of quinones.

2.4. Enzymatic modification of LNPs

The enzymatic cross-linking reactions were performed in aqueous LNP dispersions in 1.5 ml volume. The laccase activities were 500 nkat g⁻¹ (ca. 0.04 wt.-% protein) and 1000 nkat g⁻¹ (ca. 0.07 wt.-% protein) per gram of dry lignin. To obtain maximal surface and intracross-linking of a single LNP, the reactions were carried out using tenfold enzyme activities [42] i.e. 40 μkat g⁻¹ (ca. 16 wt.-% protein) for MaL and 100 μkat g⁻¹ (ca. 7 wt.-% protein) for ThL treatments. To compare cross-linking of LNPs between MaL and ThL the enzymatic reactions were carried out at equal pH 5.0 using the same enzyme dosage 40 μkat g⁻¹. Furthermore, ThL reactions were performed using enzyme dosage 100 μkat g⁻¹. After initiation of the enzymatic cross-linking reactions, the vessels were left open overnight under constant magnetic stirring to obtain the highest cross-linking density of the particles at ambient temperature. The pHs between the experiments varied from 4.3 to 5.0 for ThL and from 5.0 to 7.6 for MaL-catalyzed reactions, based on the corresponding activity profiles using guaiacol as substrate (Supplementary data Fig. A.1).

2.5. Redispersion of dried LNPs

After the enzymatic cross-linking of LNPs the dispersions were evaporated at 80 °C overnight and dried particles were redispersed in water (1.5 ml) and THF (1 ml). The reactivity of laccases was inactivated during the thermal treatment due to denaturation of the enzyme protein. The reference samples without laccases were prepared...
across-linkage of LignoBoost™ lignin by ThL and MaL, the enzymatically polymerized reaction products were analyzed by SEC. Compared to $M_n$, of the corresponding lignin controls treated without enzymes (2.8–2.9 kDa), the $M_n$ of ThL treated lignin increased 93%, whereas the $M_n$ of lignin treated with the low redox MaL, increased only 29%. Most of the polymerized lignin aggregate was insoluble in THF showing formation of covalent linkages between polymeric lignin chains.

The reactivity of ThL and MaL to cross-link single LNPs through radical generation was studied by different methods. To avoid cross-linking between single lignin nanoparticles (LNPs), low LNP concentrations (0.1–0.3 mg ml$^{-1}$) were used to restrict enzymatic re-actions only on the surface and inner part of the particles. Overnight treatments with varying enzyme activities were used to discover maximal cross-linking density of single LNPs. Oxidation of dilute LNP dispersions by the laccases was obvious since after 24 h intense, red-brown precipitates formed by the oxygen consumption measurements (Fig. 1A and 1B). Aqueous LNP dispersions treated with laccases using the high enzyme activity values for ThL and MaL, respectively, indicating evident oxidation of LNPs. The oxidation of dilute LNP dispersion with the high redox ThL was somewhat faster than when using the low redox MaL. At an equal pH 5.0 (Fig. A3, A) the difference between the reactivity of the laccases on LNPs was on the same level i.e. ca. 8 wt.% when compared to that of measured at acidic (for ThL) and alkaline (for MaL) pH (Fig. 1, A, B). The reactivity of high redox ThL and low redox MaL on lignin model compound (guaiacol) at pH 5.0 is shown also in the Supplementary materials (Fig. A3, B) for comparison to further evidence differences between the enzymes.

Zeta potential values (Table 1) and particle size distributions (data not shown) of laccase treated LNPs were measured directly from the oxygen consumption samples after finalization of the enzymatic

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2.6. Chemical oxidation of LNPs

For the chemical treatment of LNPs the pH of the dispersion (0.1 mg ml$^{-1}$) was adjusted to 9.2 with 0.1 M NaOH (ca. 0.1 mmol NaOH per gram of dry lignin) to ensure stability of the dispersion in alkaline conditions. After ultrasonication (Branson Digital Sonifier, output 400 W, equipped with a 3-mm tip, OH, USA) in the presence of 1% H$_2$O$_2$ the dispersion was divided into 10 ml aliquots and 220 µl of wt. – 33% H$_2$O$_2$ solution (60 mass equivalents of peroxide to dry lignin) was added in the reaction mixtures. The samples were stabilized (30 min) at ambient temperature and sonicated (40% amplitude) for 100 s. During the treatment, the dispersions heated up to ca. 60°C. To remove unreacted H$_2$O$_2$ from the mixtures, LNP dispersions were incubated at 60°C for 48 h. Before the average particle size and zeta potential measurements, the solvent was exchanged twice by centrifugation to remove residual H$_2$O$_2$, following redispersion of the particles in water. The reference samples were prepared accordingly.

2.7. Particle size and zeta potential measurements

Particle size distributions and zeta potential values of LNP dispersions after enzymatic and chemical treatments were analyzed using a Malvern Zetasizer Nano-ZS90 instrument (UK). The zeta potential values were calculated from the electrophoretic mobility data using a Smoluchowski model. Three to six runs from the same sample preparation were measured to evaluate the reproducibility of the measurement.

2.8. Atomic force microscopy

Atomic force microscopy (AFM) was used to characterize surface morphology and roughness of LNPs before and after the laccase treatments and redispersion of the particles in water and THF. For the imaging, 10 µl aliquot of the sample was pipetted on a freshly cleaved mica sheet and dried overnight at ambient temperature. All samples were imaged in tapping mode in ambient air using a MultiMode 8 AFM equipped with a Nanoscope V controller from Bruker Corporation (MA, USA). NCHV-A probes, with a fundamental resonance frequency of 320–370 kHz, a nominal spring constant of 40 N m$^{-1}$, and a tip radius below 10 nm (Bruker, MA, USA) were used for the imaging. At least three sample areas were analyzed from the same mica sheet, without further processing of the images except flattening using Nanoscope Analysis 8.15 software from Bruker (MA, USA).

2.9. Transmission electron microscopy

FEI Tecnai 12 (OR, USA) operating at 120 kV was used to obtain transmission emission microscopy (TEM) images from single LNPs re-dispersed in THF. For the imaging 3 µl of the sample were applied on a carbon film supported grid and incubated for 2 min. The excess of the organic solvent was removed by blotting the side of the grid onto filter paper. Imaging was performed in the bright field mode with slight under-focus.

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3. Results and discussion

3.1. Effect of thermal drying on LNPs

Aqueous LNP dispersions prepared from Kraft lignin [7] at pH 4.3 (for ThL treatment) and 7.5 (for MaL treatment) were evaporated at 80°C overnight to evaluate applicability of the moderate temperature for the concentration and drying of LNPs with and without enzymatic cross-linking. It has been shown that thermal treatments below 100°C do not affect physicochemical properties of Kraft lignin [3,4,32,43,44]. The redispersion of dried LNPs in water at slightly alkaline reaction conditions was somewhat faster than for the particles dried at acidic pH as detected by DLS (Fig. A2, B). This may be explained by the lower absolute zeta potential value of LNPs in acidic conditions. However, when compared to never-dried dispersions, the zeta potential values of dried and redispersed LNPs became slightly less negative. This small change in the zeta potential value could originate from a small reduction in the number of carboxylic acid groups on the LNP surfaces through a mild, temperature-induced decarboxylation [45]. Compared to the particle size measurements performed at pH 4.3 practically no aggregates were detected by DLS at pH 7.5 (Fig. A1, B), which is in line with the better electrostatic stability of the LNP dispersions at elevated pH, as reported previously [7].

3.2. Enzymatic oxidation and internal cross-linking of LNPs

Laccase catalyzed cross-linking reactions on technical lignins and lignin model compounds in several solvent systems have been extensively studied aiming to understand the structure-function properties of the enzymes in solution as well as at the liquid-solid interfaces [26–31]. Thus, to prove the cross-linking of LignoBoost™ lignin by ThL and MaL, the enzymatically polymerized reaction products were analyzed by SEC. Compared to $M_n$ of the corresponding lignin controls treated without enzymes (2.8–2.9 kDa), the $M_n$ of ThL treated lignin increased 93%, whereas the $M_n$ of lignin treated with the low redox MaL increased only 29%. Most of the polymerized lignin aggregate was insoluble in THF showing formation of covalent linkages between polymeric lignin chains.

Then the applicability of ThL and MaL to cross-link single LNPs through radical generation was studied by different methods. To avoid cross-linking between single lignin nanoparticles (LNPs), low LNP concentrations (0.1–0.3 mg ml$^{-1}$) were used to restrict enzymatic reactions only on the surface and inner part of the particles. Overnight treatments with varying enzyme activities were used to discover maximal cross-linking density of single LNPs. Oxidation of dilute LNP dispersions by the laccases was obvious since after 24 h intense, red-brown color (Fig. 1A and B insert), originating from the formation quinones [30] was observed.

Furthermore, the reactivity of ThL and MaL on LNPs was confirmed by the oxygen consumption measurements (Fig. 1A and 1B). Aqueous LNPs dispersions treated with laccases using the high enzyme activity values for ThL and MaL, respectively, indicating evident oxidation of LNPs. The oxidation of dilute LNP dispersion with the high redox ThL was somewhat faster than when using the low redox MaL. At an equal pH 5.0 (Fig. A3, A) the difference between the reactivity of the laccases on LNPs was on the same level i.e. ca. 8 wt.% when compared to that of measured at acidic (for ThL) and alkaline (for MaL) pH (Fig. 1, A, B). The reactivity of high redox ThL and low redox MaL on lignin model compound (guaiacol) at pH 5.0 is shown also in the Supplementary materials (Fig. A3, B) for comparison to further evidence differences between the enzymes.

Zeta potential values (Table 1) and particle size distributions (data not shown) of laccase treated LNPs were measured directly from the oxygen consumption samples after finalization of the enzymatic
enzymatic oxidation (500 nkat g\(^{-1}\)) after oxidation by ThL, and from concentration, the zeta potential changed from redox ThL than with low redox MaL. This con

aggregation of the nanoparticles occurred after the enzymatic oxidation of LNPs in dilute dispersions with high and low redox laccases. The effect of the laccase treatments on the surface morphology of LNPs was studied by AFM. Some representative images are shown in Fig. 2A–C. As reported previously [7], the surface of unmodified LNPs is smooth (Fig. 2A). A slight increase in the surface roughness (Fig. 2B) occurred after treating the LNPs with high redox ThL at low lignin nanoparticle concentration. Some aggregates (Fig. 2C) were observed in concentrated LNP dispersions oxidized with ThL likely due to the intercross-linking of the particles. Instead, when the LNP dispersions were treated with MaL using varying enzyme activities, the surface morphology of the particles remained nearly unchanged (data not shown).

No large, intercross-linked lignin particles became visible after MaL-treatments regardless of the enzyme activity and substrate concentration used in the study.

3.3. Stability of cross-linked LNPs redispersed in aqueous media

Aqueous LNPs dispersions from Kraft lignin [7] including the enzymatically oxidized lignin nanoparticles are stable at a wide pH range as explained above. However, to study stability of the enzymatically cross-linked nanoparticles in water after redispersion, the LNP dispersions at pH 4.3 and 7.5 were evaporated at 80 °C overnight and dried particles were redispersed in water. In general, MaL-treated LNPs could be easily redispersed in water (Fig. 3A). The particle size distributions were nearly identical to that of the corresponding non-treated LNPs regardless of the enzyme activity used in the study. In contrast, the redispersion of ThL-treated LNPs in water (Fig. 3B) was not very efficient, as indicated by the small shift in the particle size distributions towards larger diameters including the presence of some aggregates larger than one μm when compared to that of the corresponding reference sample. The polydispersity index (PDI) for the non-treated LNPs at different pHs as well as for MaL-treated LNP dispersions were on the same level i.e. ca. 0.20 (Table A.3). Instead, for ThL-treated LNP dispersions the PDI was somewhat higher, ca. 0.35. A comparison between the absolute zeta potential values (Table 1) reveals a systematic reduction in the values of enzymatically treated LNPs after drying and redispersion in water. A similar effect was observed with the corresponding non-treated LNPs, suggesting that decarboxylation occurred

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>LNP dispersion (mg ml(^{-1}))</th>
<th>Enzyme Dosage (nkat g(^{-1}))</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>1.0</td>
<td>ThL 500</td>
<td>-31 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ThL 1000</td>
<td>-42 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 500</td>
<td>-44 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 1000</td>
<td>-45 ± 1</td>
</tr>
<tr>
<td>B.</td>
<td>1.0</td>
<td>ThL 500</td>
<td>-37 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ThL 1000</td>
<td>-43 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 500</td>
<td>-47 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 1000</td>
<td>-44 ± 2</td>
</tr>
<tr>
<td>C.</td>
<td>0.1</td>
<td>ThL 500</td>
<td>-31 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ThL 1000</td>
<td>-36 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 500</td>
<td>-40 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 1000</td>
<td>-44 ± 2</td>
</tr>
<tr>
<td>D.</td>
<td>0.1</td>
<td>ThL 500</td>
<td>-32 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ThL 1000</td>
<td>-36 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 500</td>
<td>-40 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MaL 1000</td>
<td>-44 ± 2</td>
</tr>
</tbody>
</table>

* Group of the independent variables used for the ANOVA analyses.

Table 1 Zeta potential values of LNPs from the oxygen consumption measurement after finalization of the enzymatic reactions.

The average particle size of LNPs (ca. 200 nm) remained nearly unchanged regardless of the enzyme activity and LNP concentration used in the experiment. This indicates that no significant aggregation of the nanoparticles occurred after the enzymatic oxidation. In contrast, the absolute zeta potential value of LNPs became more negative after the laccase treatments (Table 1). At 1.0 mg ml\(^{-1}\) LNP concentration, the zeta potential changed from -31 mV to -42 mV after oxidation by ThL, and from -37 mV to -45 mV after oxidation by MaL, showing slightly more efficient oxidation of LNPs with high redox ThL than with low redox MaL. This confirms the oxidation of the LNP surfaces and the formation of novel carboxylic acid groups. More importantly, the increase in the absolute zeta potential value is an indication that the enzymatic oxidation further improves the colloidal stability of LNPs in aqueous media.

One-way ANOVA was used to confirm that addition of enzyme in the LNP dispersion changes the zeta potential value in statistically significant manner regardless of the type of the laccase and the substrate concentration. This was the case in all four treatment groups as P-values are < 0.05 (Table A.1, A). ANOVA with pooled sample verified the conclusions (Table A.1, B). Furthermore, three-way ANOVA was used to compare the groups across the different reaction conditions. With the small data set (Table 1) it was shown (Table A.2, A) that the type and enzyme dosage affect zeta potential value statistically notable manner (P-value < 0.05), however, the effect of the concentration was minor. Thus, when larger data set was used in the analysis the P-value for the concentration was also below α (Table A.2, B) confirming oxidation of LNPs in dilute dispersions with high and low redox laccases.
Fig. 2. AFM amplitude images of laccase treated LNPs. A) Reference (0.1 mg ml⁻¹, pH 4.5) B) ThL-treatments (1000 nkat g⁻¹) in 0.1 mg ml⁻¹ and C) in 1.0 mg ml⁻¹ substrate concentrations at pH 4.5. In D) is shown distribution of LNPs imaged from the same sample as particle shown in B. All images were measured in air after drying the nanoparticles on a mica sheet.

Fig. 3. Particle size distributions of LNPs (0.3 mg ml⁻¹) cross-linked with A, C) Mal. and B, D) ThL, dried and redispersed in water and in THF, respectively. Size distributions for non-treated LNPs dried and redispersed in THF are shown in Figs. A.4.6 and A.4.3. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)
during the thermal drying [44]. Instead, after enzymatic oxidation of LNPs, the zeta potential values became slightly more negative due to the formation of new carboxylic acid groups as explained above (chapter 3.1 and 3.2).

3.4. Stability of cross-linked LNPs redispersed in organic solvent

The utilization of LNPs in any water sensitive industrial applications requires that LNPs are stable also in organic solvents. This means that the particles do not dissolve or aggregate in the media. Hence, the stability of the enzymatically cross-linked LNPs was studied in THF, which efficiently dissolves Kraft lignin including unmodified LNPs.

Following a similar procedure as described above, the enzymatically cross-linked LNPs were dried and redispersed in THF for 1 and 7 days. Particle size distributions measured from THF dispersion are shown in Fig. 3C and D confirming the improved stability of the enzymatically cross-linked LNPs in organic solvent over the corresponding non-treated ones. A particle size (< 10 nm) is typical for dissolved lignin polymer. Clear correlations between the type of the laccase and enzyme activity (i.e. extent of the particle oxidation) on the stability of LNPs in THF were observed. This strongly supports the hypothesis that the LNPs were stabilized via laccase catalyzed covalent surface and intraparticle cross-links. While average particle size of ca. 570 nm (mode ca. 530 nm) was measured in THF for LNPs cross-linked with MaL at low activity, smaller average particle sizes of ca. 430 nm (mode ca. 340 nm) were measured for the particles cross-linked with tenfold enzyme activity (Fig. 3C). Similarly, average diameters of ca. 1200 nm (mode ca. 830 nm) and ca. 450 nm (mode ca. 400 nm) were obtained in THF for the particles cross-linked with low and high ThL activities, respectively (Fig. 3D). In all cases, the incubation time in THF was one day. PDI values for enzymatically cross-linked LNP dispersions were elevated varying from 0.42 to 0.86 (Table A.3). The higher particle size and PDI of LNPs redispersed in THF compared to that (ca. 200 nm) in aqueous dispersions (Fig. 3A and B) is likely due to the swelling of the particles and loosening of the spherical tightly packed structure. Instead, when LNPs cross-linked with MaL and ThL using the highest enzyme activity were incubated in THF for 7 days, the average particle size was ca. 490 nm (mode ca. 530 nm) in both cases (Fig. 3C and D, blue dashed line). Low redox MaL reactive in alkaline pH could cross-link electrostatically stable LNPs slightly more efficiently than high redox ThL reactive in acidic conditions. This indicates excellent colloidal stability of LNPs due to ionization of phenolic hydroxyl groups at slightly alkaline dispersion having, to a certain extent, a positive effect on the enzymatic radicalization of phenolic groups and covalent cross-link formation.

The phenomenon was confirmed by cross-linking LNPs with MaL and ThL at equal pH 5.0 using the high enzyme dosages. In the Supplementary material (Fig. A.4) are shown the particle size distributions measured after drying and redispersing the enzymatically cross-linked nanoparticles in THF for 1 day. In general, the average particle diameters and modes were larger than for the LNPs cross-linked at acidic and alkaline pH. With MaL, the average particle size was ca. 1230 nm (mode ca. 712 nm). Accordingly, the average diameter of ca. 700 nm (mode ca. 531 nm) was measured in THF for LNPs cross-linked with ThL using the same activity (40 μkat g⁻¹). For the particles cross-linked using the highest ThL dosage the average particle size was somewhat smaller ca. 680 nm (mode ca. 458 nm) verifying the effect of the enzyme dosing on the cross-linking efficiency. PDI values for enzymatically cross-linked LNP dispersions were on the same level with the corresponding reference (Table A.3).

The particulate morphology of LNPs after cross-linking with MaL and redispersion of the particles in THF was confirmed by AFM (Fig. 4A). Individual particles with varying sizes below 200 nm were imaged after drying of the dispersion on a mica surface. Representative high-resolution AFM images of MaL-treated and non-treated single LNP redispersed in THF for 1 day are shown in Fig. 4B, D, G and C, E, H, respectively. The lack of internal cross-linking of the non-treated LNPs made them more susceptible to collapse and dissolving in THF. The cross-section profiles of MaL and non-treated particles are shown in Fig. 4F, confirming the effect of enzymatic cross-linking on the structural integrity and improved stability of the particles in THF over the non-treated ones.

In Fig. 5 are shown, for comparison, TEM images for ThL and non-treated LNPs dried on carbon grid after 1 day incubation in THF. When the nanoparticles were cross-linked with ThL using the highest enzyme activity, spherical particles of ca. 200 nm were imaged by TEM (Fig. 5A). In addition, smaller individual particles of ca. 150 nm were detected from the carbon grid. In contrast, non-treated LNPs showed poor stability in THF. LNPs dried from the aqueous dispersion at pH 4.3 and redispersed in THF, comparison between particle size distributions (number, volume and intensity) showed dissolution and aggregation of the particles in the organic solvent. TEM image (Fig. 5B) reveals that the spherical morphology of the non-treated LNPs was nearly destroyed when the particles were incubated in THF for one day. In addition to the single broken particles, some hollow and extensively aggregated particles were observed. However, small particles ca. 10 nm (Fig. 5C) were the majority in the reference sample indicating dissolving of the non-treated LNPs in THF. Moreover, these results show that nanosized colloidal lignin particles are more difficult to dissolve in THF than the original dried LignoBoost™ lignin powder.

Fundamental understanding of the laccase-catalyzed reactions on the particulate lignin matter enables novel innovations for the use of oxidodeuctases in lignin valorization. The low redox potential MaL was slightly more efficient in the cross-linking of LNPs than high redox potential ThL. In alkaline reaction condition, the surfaces of electrostatically stable colloidal lignin particles were more prone to enzymatic radicalization and covalent cross-linking while in the acidic dispersion the surfaces of LNPs were more susceptible to aggregation.

3.5. Chemical decolourization of LNPs

The potential to exploit a mild chemical oxidant (H₂O₂) combined with ultrasonication treatment in alkaline reaction conditions [14] to decolorize LNP dispersions was investigated (Fig. 6). During the pH adjustment from 4.7 up to 9.2 of the dispersion a buffering effect arisen from the ionization of carboxylic acid and phenolic hydroxyl groups was detected as previously [27]. The amount of NaOH used to adjust the pH of the LNP dispersion was an order of magnitude lower than the theoretical quantities of the functional groups [7]. This indicates that in nanosized colloidal lignin particles the ion exchange reactions occur on the surface of the particles with limited diffusion of the hydroxyl ions inside LNPs due to the tight packing of the hydrophobic functional groups in acidic conditions. During the pH adjustment, the color of LNP dispersion darkened as shown in Fig. 6.

When the LNP dispersions at pH 4.7 and 9.2 were treated with H₂O₂, no significant changes in the colors of the dispersions were observed visually after 30 min at ambient temperature. Also after sonication, the color of LNP dispersions remained unchanged. However, when the dispersions were slightly heated (60 °C) the red-brown color decreased as a function of time. Decolorization was finished after 2 h at pH 9.2. Instead, at pH 4.7 a longer incubation time (6 h) was needed to get a corresponding effect. Finally, after 48 h thermal treatment, LNP dispersions had much lighter red-brown color compared to those of the references at both starting pHs. The decolorization effect was the most prominent when an alkaline starting pH was used. During the treatments, the pH of the dispersions varied to some extent. Regardless of the starting pH, the dispersion was acidic at the end of the treatment due to the formation of carboxylic acid groups [14].

The average particle sizes and zeta potential values of LNP dispersions after the treatments are shown in Fig. A.5. The largest change in size was observed after addition of H₂O₂ in alkaline reaction condition resulting in ca. 10% reduction in the particle size. This suggests internal cross-linking or oxidative degradation of LNPs via radical reactions.
Instead, changes in the absolute zeta potential values were more apparent at both starting pHs. Compared to the non-treated dispersions, the zeta potential values became ca. 19% and ca. 23% more negative at pH 4.7 and pH 9.2, respectively (Fig. A.5) due to the formation of carboxylic acid groups. A corresponding phenomenon was observed when LNP dispersions were oxidized with laccases (Table 1) the main difference being that H$_2$O$_2$ oxidation at elevated temperatures also induced the decolorization of the lignin nanoparticles.

The enzymatic and chemical methods described to modify LNPs are scalable, which is crucial for the applicability of the tailored particles in industrially relevant quantities. Similar laccases to the ones used in this study are commercially available at industrial scale. Hence, it is proposed that the LNP stabilization and decolorization methods demonstrated may be exploited as a final refining step when preparing LNPs from technical lignins. Thus, the advanced bionanomaterial applications of LNPs could include e.g. Pickering emulsions, foams to be exploited in medicine, food and cosmetic industries. Compared to cellulose nanocrystals it is anticipated that colloidal nanosized lignin particles could be even more attractive bionanomaterials to be exploited in such value-added applications.

4. Conclusions

It was shown that LNPs prepared from softwood Kraft lignin could be enzymatically reinforced using laccase catalyzed cross-linking reactions. These lignin nanoparticles could be redispersed in THF and water after thermal drying. As the surface and internally cross-linked LNPs retained their spherical shape and size in the organic solvent, the applicability of LNPs in industrial colloidal products as stabilizing agents, dispersants or surfactants was significantly improved. Increased use of LNPs in various formulations and matrices may be obtained via better reactivity and dispersability of nanosized lignin particles over powdered lignin. In addition to enzymatic reinforcement of LNPs, the brown color of aqueous LNP dispersions could be faded using mild chemical oxidation. The unpleasant odor, also typical for Kraft lignin was efficiently removed during the preparation of LNP dispersions.

Apparently, Kraft lignin in the form of light, stable and spherical nanoparticles in a non-malodorous colloidal dispersion in water or organic solvent is an attractive bionanomaterial for many industrial applications. Further tailoring of LNP surfaces is underway to meet the full applicability of the particles for advanced bionanomaterial applications.

Conflict of interest statement

The authors declare that there are no conflicts of interest.
Acknowledgements

This work was supported by the national institutes as Aalto University and VTT Technical Research Centre of Finland Ltd (Forest meets chemistry – program); TEKES The Finnish Funding Agency for Innovation (COLIAD-project); the Academy of Finland (grant numbers 276696, 278279, 263504, 267497, 273645); the Academy of Finland’s Centers of Excellence Programme (grant number 272361). In addition, Dr. Martina Andberg (VTT Technical Research Centre of Finland Ltd., Espoo Finland) is acknowledged for providing the laccases for the study. Päivi Matikainen and Outi Liehunen (VTT Technical Research Centre of Finland Ltd., Espoo, Finland), Marja Kirkkäinen, Rita Hatakka and MSc. student Yan Zhenxing (Aalto University, Espoo, Finland) are thanked for the laboratory assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.enzmictec.2018.01.005.

References


