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Enzymatically and chemically oxidized lignin nanoparticles for biomaterial applications

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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, *Trametes hirsuta* (ThL) and *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_2O_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.

1. Introduction

Lignin, one of the main polymers of lignocellulosic biomass, co-stream from biorefinery and paper industry, is a sustainable and renewable resource [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 [2,3]. 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 [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 [5]. In addition to its brown color and unpleasant odor [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 [4]. In the advanced applications, technical lignin is often utilized as nanoparticles to overcome these limitations [2].

Green nano- and biotechnologies are rapidly growing research fields. The use of plant polymers as nanosized additives in materials is increasing rapidly [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 [7]. However, these particles have gained considerably less attention than nanocellulose.

Several methods have been developed for the preparation of LNPs

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[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–bioorganic 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 H_2O_2 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-azinobis-(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 ultracentrifuge (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 alkali (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 ($\bar{M}_w$) 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