Preparation and Evaluation of the Free Radical Scavenging Activities of Nanoscale Lignin Biomaterials

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There is much research on nanomaterial from natural polymer because of its biocompatibility, abundance, and non-toxicity. This work is devoted to the study of free radical scavenging activities (*FRSA*) of nanoscale lignin biomaterials, which are recognized as promising natural antioxidants. The nanoscale lignin biomaterials were prepared from alkaline lignin by a solution-precipitation method with either ethylene glycol (NL1) or alkaline solution (NL2). Structural analysis of the nanoscale lignin biomaterials were conducted by Scanning electron microscopy (SEM), laser particle size analyzer, Fourier transform-infrared spectroscopy (FT-IR), potentiometric titration, and gel permeation chromatography (GPC). The results indicated that NL2 had a smaller average particle size (278±13 nm) than NL1 (375±18 nm) and contained more phenolic hydroxyl groups (2.35±0.11 mmol/g) and had a lower weight-average molecular weight (M_w=6510±320). The *FRSA* of the biomaterials towards hydroxyl free radicals were measured and compared with the alkaline lignin. Some structure-activity relationships were proposed based on the analysis of experiment data, which revealed NL2 (IC50=0.18±0.01 mg/mL) had a 3.33 fold higher activity than NL1 (IC50=0.60±0.05 mg/mL), which could be attributed to the smaller particle size, more phenolic hydroxyl group, and lower weight-average molecular weight.

Keywords: Lignin; Nanomaterial; Biomaterial; Antioxidant; Free radical

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INTRODUCTION

Antioxidants can capture or inhibit the free radicals present in living systems and foods, avoiding oxidation by donating hydrogen atoms or transferring electrons to interrupt chain reactions involving free radicals (Salem *et al.* 2014). They can prevent the loss of food color, flavor, and active vitamins content, providing the stabilization of the molecules involved in such characteristics. Antioxidants include natural and synthetic chemicals, such as certain vitamins, tocopherols, minerals, butylated-hydroxyanisole, butylated-hydroxytoluene, and tertbutylhydroquinone (Kim *et al.* 2009). The manufacture costs, potential hazard to human bodies or animals of the synthetic antioxidants, and the need for increased food additive safety give rise to an increasing demand for other natural and safe antioxidants (Valdez-Morales *et al.* 2014). There has been increasing interest in polyphenols, which have shown many favorable effects on scavenging free radicals (Grace *et al.* 2014), such as anti-oxidation of proteins, food lipids (Kortenska *et al.* 2002), anti-inflammatory effect, and anti-carcinogenesis (Miyake *et al.* 1999).

Lignin is the most abundant natural phenolic polymer. It is crosslinked by *p*-hydroxyl phenylpropane units (*p*-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol),

composing up to one-third of the material of the plant kingdom (Ge *et al.* 2014). Alkaline lignin (AL) is obtained as a by-product from black liquor, a waste discharged from paper mills in large quantities which can pose a major problem of disposal. To date, the main use of AL is as a low-grade energy source in combustion (Ge *et al*. 2014). Although there are some other marginal applications, such as biosorbents (Ge *et al*. 2014), dispersants (Li *et al*. 2011), or adhesives (Thakur *et al*. 2014), no major large-scale application has so far been found. A new possible application for excess lignin is as a precursor for natural, safe antioxidant production (Thakur *et al*. 2014; Valdez-Morales *et al*. 2014). In fact, as a natural polyphenol polymer, lignin is a non-toxic, biodegradable, and biocompatible substance that can reduce free radicals and stabilize oxidation reactions (Li and Ge 2012; Ge *et al.* 2014; Hussin *et al.* 2014; Thakur *et al.* 2014).

Recently, there is a growing interest in using biopolymers to create value-added functional materials, such as nanoscale biomaterials. The nanoscale biomaterials' applicative impact is especially important in a wide range of fields, such as medicine, drugs, coatings, IT, auto, chemicals, cosmetics, and food industries (John and Vemula 2006; El Kadib *et al.* 2014). Lu *et al.* (2012) prepared a nanoscale lignin from organosolv lignin by a supercritical antisolvent (SAS) method; this approach had high cost and was time-consuming and not feasible for large scale production. Frustratingly, despite the abundance and wide availability of AL from industries, so far there have been only limited reports about nanoscale antioxidants produced from AL. Therefore, in this work, nanoscale lignin (NL) was fabricated from AL through a simple solution-precipitation method, and their structures were characterized by scanning electron microscopy (SEM), laser particle size analyzer, Fourier transform-infrared spectroscopy (FT-IR), and gel permeation chromatography (GPC). The antioxidant activities of the NL for scavenging hydroxyl free radicals were also investigated.

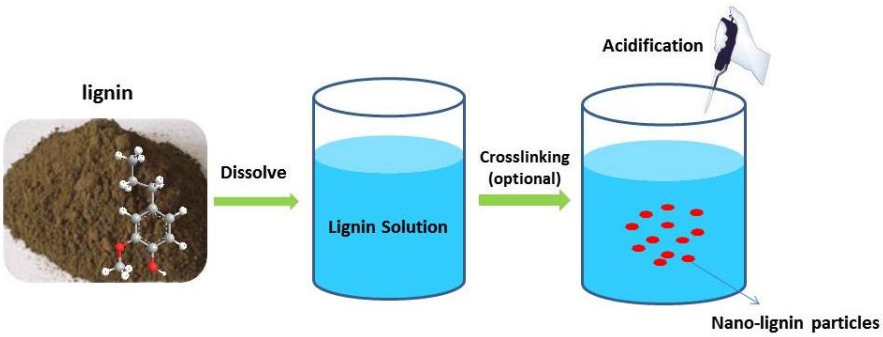
EXPERIMENTAL

Materials

Alkaline lignin was obtained from Nanpu-pulping Company, China. It was purified by an acid precipitation method as described in our previous study (Li and Ge 2011). Hydrochloric acid, nitric acid, and sodium hydroxide, ethylene glycol, and glutaraldehyde were purchased from Laotian Chemicals, China.

Synthesis of NL

NL was prepared by a simple solution-precipitation method (as shown in Scheme 1). NL1: 5.0 g AL was dissolved in 100.0 mL of ethylene glycol and was stirred for 2.5 h. 4.4 mL HCl (0.1 M) was added to the lignin solution. 150.0 mL 0.4% glutaraldehyde aqueous solution was added into lignin solution for cross-linking for 2.5 h. After the reaction was completed, the solids were separated by centrifugation at 15000 rpm and washed twice with distilled water and then dried at 55 ^{o}C under vacuum overnight. NL2: 10.0 g AL was mixed with 75.0 mL of distilled water and 25.0 mL of 1M NaOH under stirring for 15 min. An additional 5.0 mL of 1.0 M NaOH were added to the solution in 0.5 mL portions to pH=11.5. Then 15.0 mL of distilled water was added. Finally, 0.25 M HNO_3 solution was added rapidly to pH=1.9. The product was obtained by centrifugation at 15000 rpm and washed twice with distilled water and then dried at 55 ^{o}C under vacuum overnight.



Scheme 1. Preparation diagram of NL biomaterials from AL

Characterization

The surface morphologies of the samples were determined using scanning electron microscopy (SEM) (Hitachi SU8020). The particle size distribution of the samples was recorded on a Malvern Zetasizer Nano S Particle Sizer. FT-IR was recorded between 4000 and 400 cm^{-1} on a FT-IR spectrophotometer (Thermo Nicolet 510) with the KBr plate method. The quantities of carboxyl groups and Ph-OH were determined with a potentiometric titration method as described in our previous study (Li and Ge 2012), using a Metrohm 809 Titrando device. The sample was dissolved in DMF and was titrated by tetra-butyl-ammonium hydroxide with p-hydroxybenzoic acid as an internal standard. The number- and weight-average molecular weight (M_n and M_w) of lignin were measured with an Agilent1100 GPC equipped with an autosampler, an isocratic pump, a thermostated column compartment, and a multiple wavelength detector. HPLC-grade tetrahydrofuran was used as solvent and eluent. Polystyrene was used as standards (Sigma-Aldrich).

Antioxidant Activity

The hydroxyl radical can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation), and amino acids. It has a very short *in vivo* half-life of approximately 10^{-9} seconds and a high reactivity. This makes it a very dangerous compound to the organism. The antioxidant activities of lignins were determined by scavenging hydroxyl free radicals (·OH) generated by Fenton's reagent. The ·OH could oxidize salicylic acid to 2,4-dihydroxy-6-methylbenzoic acid with an absorbance at 510 nm (Li and Ge 2012), which was measured using a UV-vis spectrophotometer.

In a typical experiment salicylic acid was added into Fenton's reagent solution and kept for 30 min at room temperature. The absorbance at 510 nm (A_0) was measured via UV spectrophotometer. Another part salicylic acid with Fenton's reagent with a certain amount of lignin was also prepared and kept at room temperature for 30 min. The absorbance at 510 nm was recorded (A_1). As expected, the absorbance decreased, which provided a way to evaluate the antioxidant activity. Additionally, the absorbance of lignin itself should be subtracted (A_L). The experiment was carried out three times. The free radical scavenging activity (*FRSA*) could be calculated by the following equation:

$$FRSA\,(\%) = \left(\frac{A_0 - (A_1 - A_L)}{A_0} \right) \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Characterization

SEM images (Fig. 1) show significant differences in the morphologies of AL and NL. The NL consisted of square-shaped nanoparticles, while the AL was in the form of irregular blocks.

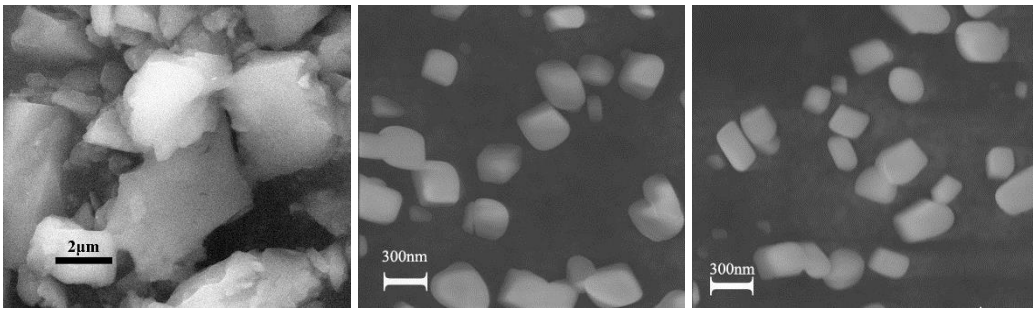


Fig. 1. SEM images of lignin biomaterials: alkaline lignin (a), NL**1** (b), NL**2** (c)

The particle size distribution is demonstrated in Fig. 2(A), which exhibits relatively normal distributions of the nanoparticles. The average particle size of NL1 was 375±18 nm, which was larger than NL2 (278±13 nm). It can also be found from Fig. 2(A) that the NL2 had a relatively narrow distribution. That was attributed to the cross-linking of lignin molecules with glutaraldehyde.

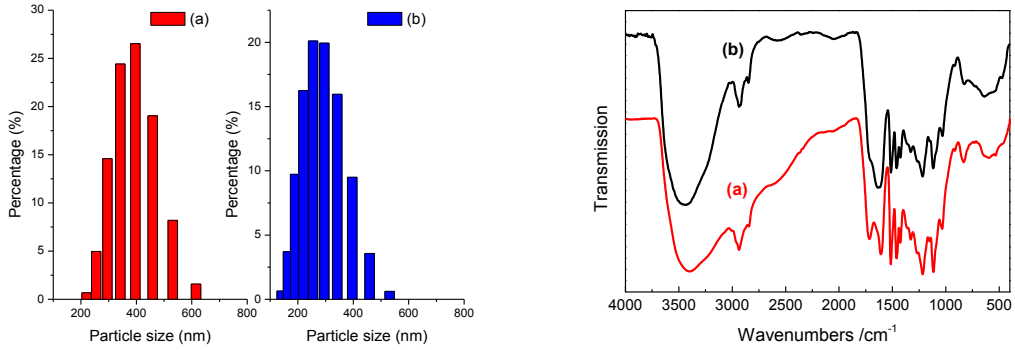


Fig. 2. Particle size distribution (A) and FT-IR spectra (B) of nanoscale lignin: NL1 (a) and NL 2 (b)

FT-IR spectra of the nanoscale lignin are shown in Fig. 2(B). These curves present typical absorption bands of lignin. The bands at 2850 and 2930 cm^{-1} are assigned to the C-H stretching of methyl and methylene groups. The bands at 1610, 1515, and 1460 cm^{-1} assigned to aromatic skeletal vibration (Hergert 1971) confirmed that the "core" of the lignin structure did not change during the preparation. The bands at 1320, 1215, and 1116 cm^{-1} are due to the C-O stretching of ether groups. The C-H in-plane deformations in aromatic rings at 830 cm^{-1} were also found. The important differences of (a) and (b) lie in the bands of 1716 cm^{-1} which was due to the crosslink of lignin and glutaraldehyde that introduced C=O groups into lignin.

The contents of carboxyl and Ph-OH groups of lignin are tabulated in Table 1. As expected, the contents of Ph-OH and carboxyl groups of NL1 were less than AL and NL2. That is attributable to the condensation polymerization between lignin and glutaraldehyde, and it also resulted in an increase of M_w. Little differences between NL2 and AL were observed, indicating no significant chemical reaction taking placed during the process.

Table 1. Properties of the Lignin Biomaterials

	Carboxyl groups (mmol·g^{-1})	Ph-OH groups (mmol·g^{-1})	M_w	M_n	M_w /M_n
AL	2.79±0.19	2.30±0.18	6750±330	2410±120	2.80
NL1	2.50±0.23	2.06±0.12	7520±390	2890±140	2.60
NL2	2.68±0.21	2.35±0.11	6510±320	2600±130	2.50

Antioxidant Activity

Figure 3 shows *FRSA* of the lignin biomaterials. There was a significant relationship between *FRSA* and the dosage of lignin. With the dosage of lignin increasing, *FRSA* increased too. For screening purposes, the IC50 (dosage of lignin at *FRSA*=50%) of each lignin was calculated. A higher antioxidant activity results in a lower IC50 value. The results indicate that NL2 was the most effective lignin fraction tested for scavenging hydroxyl free radicals, as its IC50 was 0.18±0.01 mg/mL, which is lower than alkaline lignin (0.30±0.02 mg/mL) and NL1 (0.60±0.05 mg/mL). IC50 values of the lignin biomaterials were in an acceptable range for a potent antioxidant (Pan *et al.* 2006; Ugartondo *et al.* 2008; Li and Ge 2012). This indicates that the nanoscale lignin biomaterials were of higher potency as natural antioxidants. The variance in the scavenging activity depends on the hydrogen-donating or electron-donating ability of structural characters of lignin (Dizhbite *et al.* 2004). NL2, which exhibited the higher *FRSA*, bears (Table 1) a higher quantity of Ph-OH and lower M_w.

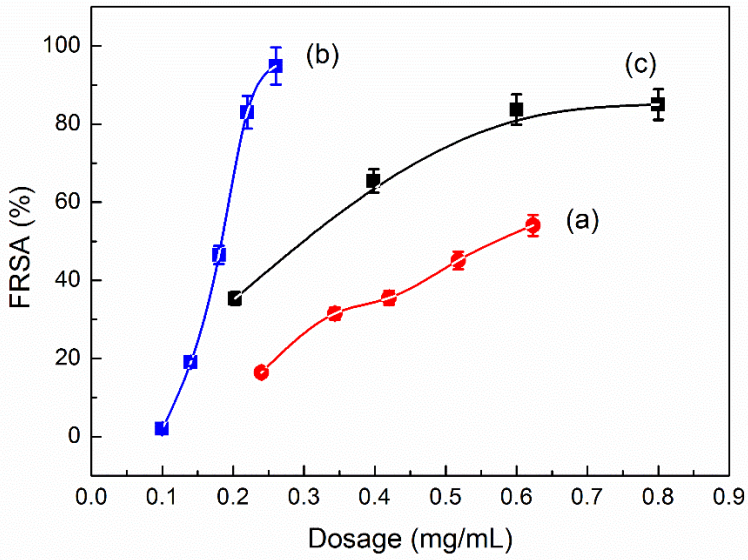


Fig. 3. Hydroxyl free radical scavenging activities of NL1 (a), NL2 (b), and AL (c)

Low molecular weight resulted from extensive depolymerization of the lignin, *i.e*., cleavage of ether linkages, which led to the formation of new Ph-OH groups, the center responsible for the trapping of radicals. In other words, the low molecular weight fraction of the lignin possessed more Ph-OH than the high molecular weight fraction (Pan *et al.* 2006). This was in accordance with the literature (Barclay *et al.* 1997; Ogata *et al.* 1997).

It is important to note that the AL, which had similar values of Ph-OH and M_w but a higher quantity of carboxyl groups compared with NL2 (Table 1), exhibited lower radical scavenging activity (IC50=0.30±0.02 mg/mL). The fact that the AL had the lower activity suggests that the carboxyl group, which is a strong electron-attracting group and will influence the electron-donating ability of lignin, can decrease the antioxidant activity of lignin. In addition, a relationship between particle size and FRSA can be observed between NL1 and NL2. That is, the smaller NL2 leads to a higher FRSA, which is due to the faster diffusion of the smaller particles.

In general, these data imply that a higher quantity of Ph-OH, smaller particle size, less quantity of carboxyl groups, and lower M_w will positively influence the antioxidant activity of nanoscale lignin biomaterials. Moreover, the lignin biomaterials showed no harmful effect on eyes and skin, as reported by Vinardell *et al*. 2008. The high antioxidant capacity of the nanoscale lignins studied, together with their safety, open new perspectives in their potential use in cosmetic, pharmaceutical, and food industries.

CONCLUSIONS

1. Nanoscale lignin biomaterials were prepared from alkaline lignin by a simple method with alkaline solution or ethylene glycol solution that was feasible for large scale production.
2. The evaluation of antioxidant activities towards hydroxyl free radicals indicated that NL2 (prepared from alkaline solution) showed higher antioxidant activity than NL1 (prepared from ethylene glycol solution) and alkaline lignin, which was due to its structure features, *i.e.* more Ph-OH, lower M_w, and smaller particle size.
3. The finding of nanoscale lignin biomaterial as a naturally occurring antioxidant can lead to promising applications in the cosmetic, pharmaceutical, and food industries.

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