# Horseradish Peroxidase Modification of Sulfomethylated Wheat Straw Alkali Lignin To Improve Its Dispersion Performance

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S *Supporting Information*
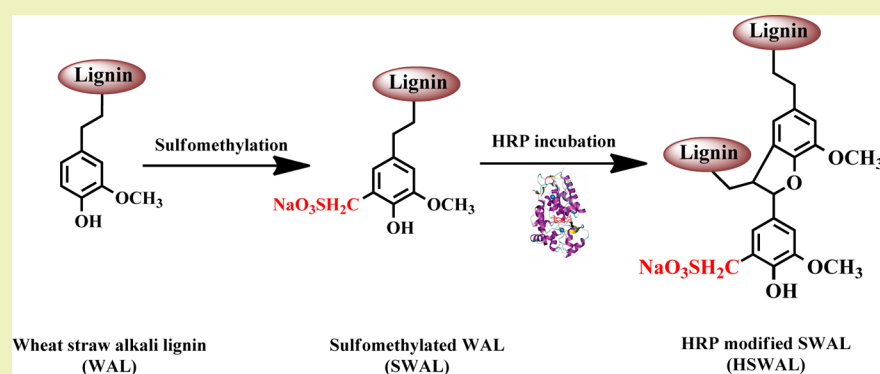
ABSTRACT: Wheat straw alkali lignin (WAL), byproducts from the alkali pulping process, is a low-value product with poor water solubility and limited dispersion performance. Sulfomethylated wheat straw alkali lignin (SWAL) was first prepared by sulfomethylation. In order to further improve the dispersion performance of WAL, a commercially available horseradish peroxidase (HRP) was then used to modify SWAL. Gel permeation chromatography showed an obvious increase in molecular weight after HRP modification by approximately 6 fold and 18 fold, compared with SWAL and WAL, respectively. The structural characterization was investigated by functional group content measurements and IR and ^{1}H NMR analyses. After the HRP modification, the phenolic and methoxyl group content decreased, while the sulfonic and carboxyl group content increased. Because of the higher molecular weight and hydrophilic group content, the HRP modification induced a significant improvement in adsorption and dispersion performance of WAL.

KEYWORDS: Horseradish peroxidase, Wheat straw alkali lignin, Molecular weight, Structural characterization, Adsorption and dispersion performance

INTRODUCTION

The soda pulping process is widely used in Chinese paper mills. Alkali lignin is the main component in this process, accounting for nearly 85% of all technical lignin produced in the world. However, more than 90% of alkali lignin is discarded as wastewater or burnt without reutilization. This is not only a waste of resource but also a tremendous pressure on the environment.[1,2] During the soda pulping process, the lignin is highly fragmented and insoluble in water at neutral pH,[3] resulting in very little alkali lignin used as industrial products. Therefore, in order to utilize the alkali lignin efficiently, it is necessary to improve its molecular weight and water solubility by modifications.

Chemical modifications, such as sulfonation,[4] oxidation,[5] and copolymerization,[6] are the main method to improve the applications of alkali lignin. The modified alkali lignin could be used as a dispersant or water-reducing agent in different industrial fields.[4,7] Among all chemical modifications, sulfome-

thylation is one of most widely used ways. The introduction of sulfonic groups improves the water solubility of alkali lignin. The sulfonic group content has a great impact on the properties of sulfomethylated alkali lignin. In our earlier report,[4,8,9] the lignosulfonate with higher sulfonic groups had better dispersive ability of coal water and cement. The molecular weight of sulfomethylated alkali lignin is also an important aspect of its function as an excellent dispersant or plasticizer. Previous studies showed that increasing the molecular weight of lignosulfonates obviously improved the dispersion performance of coal water slurry and cement particles.[8,10] However, due to the destruction of the lignin structure in the violent conditions of the soda pulping process, the reactivity of alkali lignin decreases extremely.[11] Therefore, the increase in sulfonation

degree and molecular weight is very limited during the sulfomethylation of alkali lignin.

Recently, there has been increasing interest in biological modification of lignin. The advantages of biological modification include high selectivity, high efficiency, and mild reaction conditions. Our previous studies[11,12] showed that laccase modification could improve the sulfomethylation reactivity of alkali lignin, resulting in the higher sulfonic group content. On the other hand, when laccase was used to modify sodium lignosulfonate, its molecular weight increased by 40%.[13] Therefore, we hypothesize that there may be an enzyme that could increase the molecular weight and also the hydrophilic groups content.

Horseradish peroxidase (HRP) is one of the most widely used peroxidases in various fields.[14] It has been demonstrated that HRP can oxidize and polymerize lignin model compounds and fragments.[15,16] Furthermore, according to the study by Grönqvist et al.,[17] HRP showed a higher catalytic activity than laccase in the polymerization of lignin. Recently, we developed and reported a novel enzymatic modification, an HRP−H_2O_2 system incubation.[18] We demonstrated that HRP could polymerize lignosulfonate efficiently and does not reduce the sulfonic group content. Moreover, when we used HRP to polymerize the sulfomethylated pine alkali lignin, the molecular weight and sulfonic group were both increased.[19,20] The chemical structure and content of the lignin from woody biomass is very different with that from herbaceous biomass. The difference will further affect the reactivity and application properties. As reported,[21] the sulfomethylation reactivity of pine kraft lignin was higher than wheat straw alkali lignin (WAL). When poplar, straw, bamboo, and bagasse sodium lignosulfonates were used as dispersants of coal water slurry, straw sodium lignosulfonate showed dilatant characteristic, weaker thixotropy, and worse stability.[22,23]

To further understand the fundamentals of HRP modification, we used wheat straw alkali lignin (WAL) as raw material in this work, an herbaceous biomass that had lower reactivity than woody biomass. WAL was first sulfomethylated to obtain the sulfomethylated wheat alkali lignin (SWAL) with good water solubility, and then SWAL was modified by HRP. The structural characterization changes during the reaction were investigated. In addition, the adsorption and dispersion performance of the prepared product were also reported.

■ MATERIALS AND METHODS

Materials. Wheat straw alkali lignin (WAL) was supplied by Quanlin Paper Making Co., Ltd. (Shandong, China). WAL was isolated from wheat straw alkali pulping black liquor and purified by acidic treatment with H_2SO_4. 2,2′-Azino-bis (3-thylbenzothiazoline-6-sulfonate) (ABTS), Folin Ciocalteau's phenol reagent (2 N), vanillin, hydroiodic acid (57%), methyl iodide, and poly-(diallyldimethylammonium chloride) (PDAC, M_w of 200,000−350,000, 20% solution) were purchased from Sigma-Aldrich (Shanghai, China). All of the other chemicals were of analytical grade.

Horseradish peroxidase (HRP) was supplied by Xueman Co., Ltd. (Shanghai, China). The activity of HRP, determined in accordance with the method reported by Childs et al.,[24] was 12.7 U/mg.

Sulfomethylation of WAL. Three grams of WAL was first dissolved in 10 mL of 10% sodium hydroxide solution in a reaction vessel and heated to 70 °C. Subsequently, 0.41 g of formaldehyde aqueous solution of 37% concentration was added and stirred for 1 h at 600 rpm and heated to 95 °C. Then, 0.9 g of sodium sulfite was added and reacted for another 3 h. At last, the product was adjusted to pH 6.0 with HCl solution and filtered by Büchner funnel with Whatman filter paper. The filtrate was freeze-dried (ALPHA1−2 LD plus freeze drier, Christ Corp., Germany) to get sulfomethylated wheat straw alkali lignin (SWAL).

Modification of SWAL by HRP. One gram SWAL was dissolved in 50 mL phosphate buffer (100 mM, pH 6.0) in a reaction vessel prior to addition of 1 g/L or 6 g/L (635 or 3810 U) HRP. The reaction was started by adding 0.88 mM H_2O_2 under stirring at 480 rpm at 30 °C. After the HRP incubation for 2 h, all samples were immediately heated in boiling water for 10 min to stop the reaction. The samples were then filtered by a Büchner funnel with Whatman filter paper, and the filtrates were freeze-dried (ALPHA1−2 LD plus freeze drier, Christ Corp., Germany) to obtain HRP modified wheat straw alkali lignin (HSWAL-1 or HSWAL-6).

Gel Permeation Chromatography (GPC). Aqueous GPC was conducted using Ultrahydrogel 120 and Ultrahydrogel 250 columns in series and measured with a Waters UV detector 2487 at 280 nm (Waters Co., U.S.A.). Polystyrenesulfonates with molecular weights ranging from 500 to 10 000 g/mol were used for calibration. A 0.10 M $NaNO_3$ solution (pH 10.0, adjusted with 20 wt % NaOH solution) was used as eluent at 0.50 mL/min. All samples used for GPC analysis were ion-exchanged to remove the impurities and then filtered by a 0.22 μm syringe filter. Then, 50 μL filtered samples were injected into the column for GPC analysis.

Functional Group Content Measurements. The sulfonic and carboxyl group contents of lignin were measured by automatic potentiometric titrator (905 Titrando, Metrohm Corp., Switzerland) as reported previously.[4,25] All samples were purified by ion-exchange before the measurement.

The methoxyl content of lignin was determined by the headspace gas chromatography (HS-GC) as described by Li et al.[26] All measurements were carried out using a DK-3001A headspace sampler (Beijing Zhongxing Huili Science & Technology Co., Ltd., Beijing, China) and Model GC9800 capillary gas chromatography (Kechuang Co., Shanghai, China). The conditions and procedures were similar as described previously.[18]

The phenolic group content was determined as de Sousa et al. reported.[27] Vanilla was used as the standard. The absorbance at 760 nm was measured by a UV−vis spectrophotometer (UV-2450, Shimadzu Co., Kyoto, Japan).

Spectroscopy Analyses. The IR spectra of lignin were recorded by a Nexus spectrometer (Thermo Nicolet Corp., U.S.A.) with the frequency range from 4000 to 400 cm^{-1}. Tablets were prepared by mixing 2 mg of dried samples with 200 mg of KBr (for spectroscopy) in an agate mortar. The resulting mixture was successively pressed at 12 MPa for 2 min. Then, the tablet was used for IR analysis. The spectra were normalized by Omnic software.

Thirty milligrams of lignin was dissolved in 0.5 mL DMSO-d_6. The ^{1}H NMR spectra of lignin were obtained on a Bruker DRX-400 spectrometer (Bruker Corp., Germany). The spectra were recorded at 30 °C after 32 scans. A 60° pulse angle and 6 s pulse time were used.

Preparation and Characterization of Self-Assembly Film. The adsorption performances of the lignin samples were measured by self-assembly film. SWAL or HSWAL-6 was dissolved in double distilled water directly to obtain a 1 g/L polyanionic solution. Diluted HCl was used to adjust the solution pH at 3.0. PDAC solution was used as a polycation with a concentration of 0.1 mM. According to the method reported by Deng et al.,[28] the layer-by-layer self-assembly films of lignin were prepared. The adsorption amount was detected by UV−vis spectrophotometer (UV-2450, Shimadzu Co., Kyoto, Japan) at 280 nm. The morphological images of the films were observed using a Pico Force atomic force microscope (AFM) (Veeco, Corp., U.S.A.) by the tapping mode. Software XEI 1.8.0 Build20 (Veeco, Corp., U.S.A.) was used for calculating a root-mean-square height to determine the surface roughness of the lignin-coated surface.

Dispersion Performance. The dispersion performance of lignin on TiO_2 slurry was measured by Turbiscan Lab Expert (Formulaction, France). The 0.5 wt % lignin samples were slowly mixed with 3 wt % of TiO_2 powder and stirred at 300 rpm for 15 min before measurement. The Turbiscan technology consists in measuring backscattering and transmission intensities versus the sample height (~40 mm) as functions of time (60 min). Through using the TLAB

EXPERT software, the variations of TiO_2 particle diameter in the opacity zone can be calculated.

■ RESULTS AND DISCUSSION

Molecular Weight Distribution and Sulfonation Degree of SWAL Modified by HRP. It is said that the molecular weight and sulfonation degree of sulfonated lignin has a significant influence on its applications.[8,29] Lignosulfonates with higher molecular weight and sulfonic group content had better dispersive ability of coal water slurry and cement.[4,9] The molecular weight distribution and sulfonic group content of lignin are shown in Figure 1 and Table 1. After
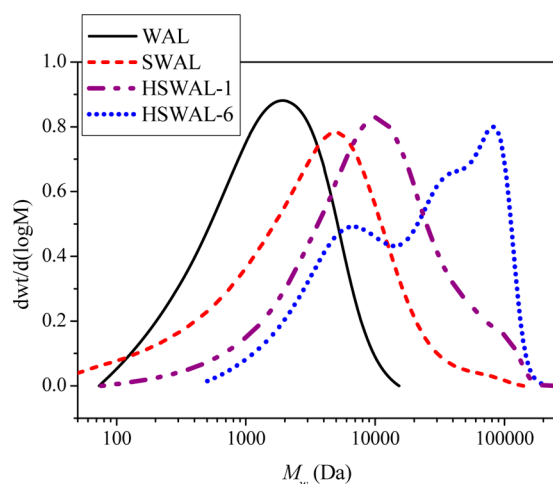

Figure 1. Molecular weight distribution of WAL, SWAL, HSWAL-1, and HSWAL-6.

Table 1. Molecular Weight Distribution and Sulfonic Group Content of Lignin Samples

	M_n (Da)	M_w (Da)	M_w/M_n	sulfonic group content (mmol/g)
WAL	900	1900	2.05	−
SWAL	1700	6300	3.76	1.97
HSWAL-1	3500	17400	5.02	2.10
HSWAL-6	7700	35700	4.65	3.05

sulfomethylation, the sulfonic group content of SWAL was 1.97 mmol/g, and the molecular weight of SWAL was 6300 Da compared with the 1900 Da of WAL. To further improve these characterizations, HRP modification was carried out for SWAL. Compared with SWAL, there was a significant increase in the molecular weight of HSWAL. As for HSWAL-6, the molecular weight increased by 470% (35,700 Da). Furthermore, a new peak ($M_p \approx 80,000$ Da) appeared in the distribution curve of HSWAL-6 (Figure 1). This indicated that HRP could polymerize sulfonated lignin efficiently. Simultaneously, the sulfonic group content increased by 55%, reaching 3.05 mmol/g. This might be due to the reaction between the radicals produced by HRP oxidation with the residual sodium sulfite. As shown in Table 1, the higher the HRP concentration is, the higher the improvement in HSWAL is.

Chemical modifications could also increase the molecular weight and sulfonic group content.[30,31] According to Yi et al.,[31] when calcium lignosulfonate is used as the raw material to undergo oxidation, sulfomethylation, and the chelation reaction, the molecular weight and sulfonic group content of calcium lignosulfonate could increase approximately 3-fold and

by 24%. However, the increase was still lower than that from HRP modification.

Functional Groups Contents. The study of the functional group content changes during the modification is helpful for understanding the reaction mechanism. After sulfomethylation, the other functional groups contents were almost unchanged except for the introduction of the sulfonic group (Figure 2 and
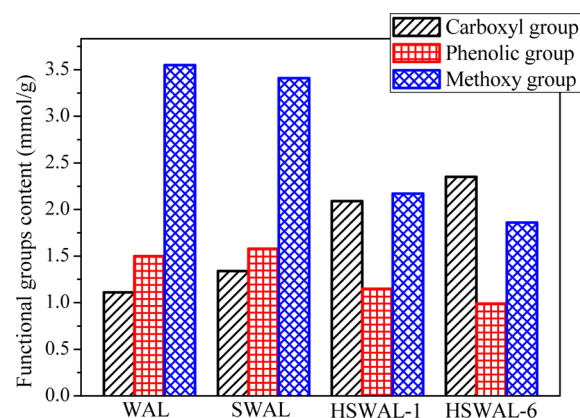

Figure 2. Functional groups contents of WAL, SWAL, HSWAL-1, and HSWAL-6.

Table 1). However, the functional groups changed a lot after the HRP modification. HRP could oxidize the phenolic groups to produce phenoxy radicals. These radicals underwent random radical couplings, resulting in an increase in molecular weight. Therefore, the phenolic group content decreased significantly, especially for HSWAL-6 (Figure 2). HRP also oxidized hydroxyl and aldehyde groups of SWAL into carboxyl groups. Compared to SWAL, the carboxyl group content of HSWAL-1 and HSWAL-6 increased by 56% and 75%, respectively. On the other hand, the methoxyl group content decreased after HRP modification, suggesting that there existed some demethylation. The changes in the functional groups contents will affect the application performance of lignin samples. We will discuss the application performance later.

IR Analysis. The structural characterization was also investigated by IR spectroscopy.[32,33] As observed in Figure 3,
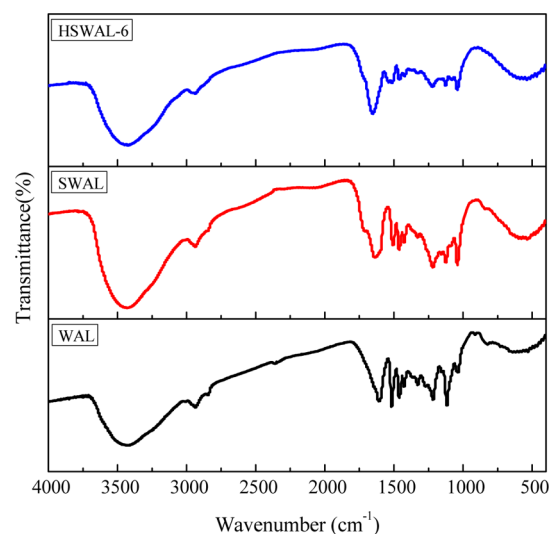

Figure 3. IR spectra of WAL, SWAL, and HSWAL-6.

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Table 2. Signal Assignment and Intensity of ^{1}H-NMR Spectra of WAL, SWAL, and HSWAL-6

		intensity		
δ (ppm)	assignments	WAL	SWAL	HSWAL-6
7.80−7.25	H_2, H_5, H_6 in hydroxy-phenyl units (H)	1.15	1.46	1.42
7.25−6.80	H_2, H_5, H_6 in guaiacyl units (G)	1.28	1.51	0.68
6.80−6.25	H_2, H_6 in syringyl units (S)	1.36	1.09	0.75
6.25−5.75	H_α in β-O-4′ and β-1′ structures	0.41	0.83	0.86
5.75−5.24	H_α in β-5′ structures	0.56	1.16	1.35
4.90−4.43	H_α, H_β in β-O-4′ structures	0.81	2.34	2.15
4.30−4.00	H_γ in β−β′ structures	0.66	2.24	2.08
4.00−3.20	Protons in methoxyls	7.75	6.42	5.61
3.20−3.00	H_β in β−β′ structures	0.86	0.65	0.53
2.60−2.40	DMSO	1.00	1.00	1.00
1.60−0.38	H in aliphatic acetates	3.07	3.18	3.97

a broad peak focused at 3435 cm^{-1} was attributed to the stretching vibration of aromatic and aliphatic OH groups. Bands near 2938 and 2850 cm^{-1} represented the C−H stretching vibration of the −CH$_2$, −CH$_3$, and −OCH$_3$ groups. The absorbance at 1705 cm^{-1} corresponds to carbonyl stretching in unconjugated ketones. The multibands between 1620 and 1420 cm^{-1} were assigned to aromatic skeletal vibrations in lignin. The band near 1328 cm^{-1} was due to syringyl ring breathing with C−O stretching vibration. The peak near 1116 cm^{-1} was assigned to syringyl ring breathing. The peak near 1215 cm^{-1} was due to the C−O stretching vibration of phenolic hydroxyl and phenolic ether. The band near 1120 cm^{-1} was attributed to the aromatic C−H in-plane deformation. The band at 1040 cm^{-1} was attributed to the S═O stretch in the sulfonic group.

The peak around 2850 cm^{-1} became less apparent after sulfomethylation and even disappeared after HRP modification (Figure 3). It was suggested that there might be some demethylation during the reaction. Compared with WAL, a new peak at 1705 cm^{-1} appeared in the spectrum of SWAL and then disappeared in HSWAL-6. Probably it was due to the C═O vibration of the unreacted formaldehyde in SWAL. After the HRP modification, the disappearance of this band might be attribution to the further reaction of SWAL with formaldehyde, activated by HRP oxidation. This opinion could also been supported by a further increase in the sulfonic group content in HSWAL, as shown in Table 1, and also an increase in the band at 1040 cm^{-1} (Figure 3).

^{1}H NMR Analysis. In order to further investigate the influence of sulfomethylation and HRP modification on lignin, the semi-quantitative method of ^{1}H NMR was used to analyze the structural changes of lignin (Figure S1, Supporting Information). The intensities of different regions, shown in Table 2, were normalized to the DMSO-d_6 cross-signal (2.60−2.40 ppm).

The signals around 7.80−7.25, 7.25−6.80, and 6.80−6.25 ppm were attributed to protons in hydroxy-phenyl (H), guaiacyl (G), and syringyl (S) units, respectively.[34] The decrease in S units, especially for HSWAL-6, was due to the demethylation during HRP modification, resulting in an increase in H units. This was in good agreement with the decrease in the intensity of protons in methoxyls (4.00−3.20 ppm). However, the amount of G units decreased after HRP modification. It was suggested that G units might be the main reaction point during the HRP modification. The signals around 4.90−4.43, 4.30−4.00, and 3.20−3.00 ppm, assigned to H_α, H_β in β-O-4′ and H_γ and H_β in β−β′ structures,[35]

decreased after HRP modification, which indicated that β-O-4′ and β−β′ might be broken. However, the signals (5.75−5.24 ppm) attributed to H_α in β-5′ structures increased after HRP modification. It indicated that the main linkage of radicals coupling was β-5′ bond during HRP modification. In terms of SWAL compared with WAL, the signals between 6.25 and 4.00 ppm were all increased (Table 2), which might be due to the increase in molecular weight after sulfomethylation. Owing to the complicated structure of natural lignin, further investigations using lignin model compounds are in progress to confirm the hypothesis above.

Adsorption Characterization. The SWAL/PDAC and HSWAL-6/PDAC layer-by-layer self-assembly films were prepared at pH 3.0 by alternately adsorbing SWAL or HSWAL-6 with PDAC. The absorbance at 280 nm of each layer was recorded to evaluate the adsorption amount of the lignin samples. As presented in Figure 4, a linear increase in
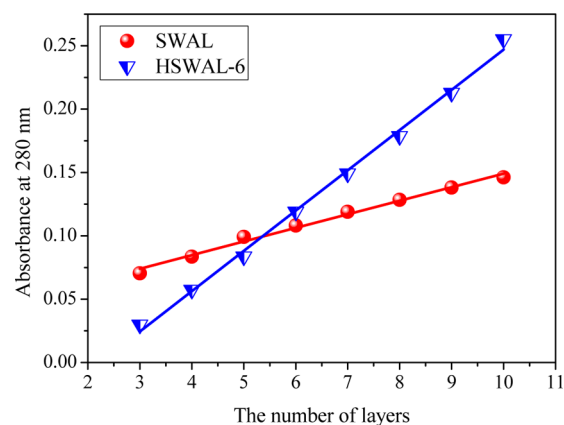

Figure 4. Absorbance of SWAL/PDAC and HSWAL-6/PDAC multilayers at 280 nm varying with bilayer numbers.

absorbance at 280 nm with an increasing number of bilayers could be observed except for the first three bilayers because of a substrate effect. After the HRP modification, the adsorption amount increased significantly. This was mainly due to the increased molecular weight and sulfonic group of HSWAL-6, resulting in higher hydrophobic interaction and electrostatic attraction during self-assembly. Thus, HRP modification could significantly improve the adsorption performance of SWAL.

The morphology of SWAL/PDAC and SWAL/PDAC multilayers with SWAL or HSWAL-6 as the outmost layer was observed by AFM (Figure 5). The root-mean-square
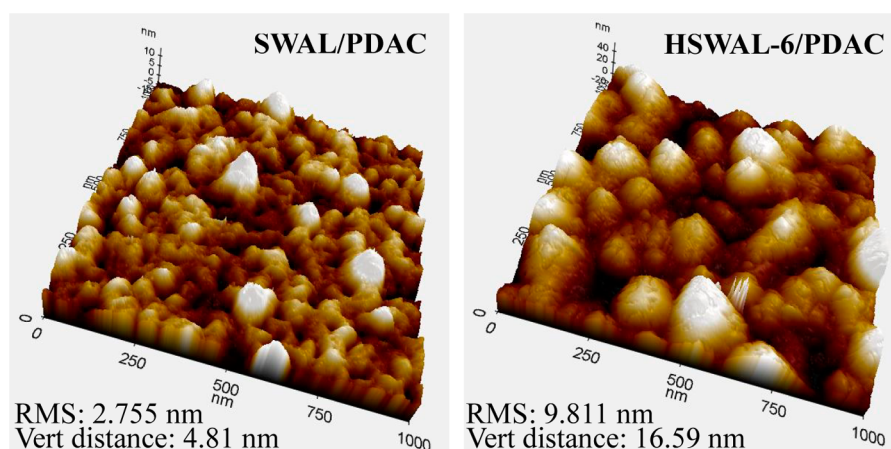
Figure 5. AFM images of the tenth bilayer of the SWAL/PDAC and HSWAL-6/PDAC self-assembly film with SWAL or HSWAL-6 as the outmost layer.

roughness (RMS) and typical vertical distance of the SWAL/PDAC bilayer were 2.755 and 4.81 nm, respectively. Nevertheless, after HRP modification, the RMS and typical vertical distance of the HSWAL-6/PDAC bilayer increased to 9.811 and 16.59 nm, respectively. In addition, the peak of the HSWAL-6 macromolecule was broader and bigger than that of SWAL.

Dispersion Performance. As is known, TiO_2 is one of the most widely used mineral oxides in industry. The applications of TiO_2 include paints, plastics, papermaking, ceramics, fibers, and inks.[36,37] The dispersion stabilization of TiO_2 is very important for these industries. Because of their large surface area and surface properties, TiO_2 particles often tend to form agglomerates during the process, leading to the end-use properties being highly affected. As shown in Figure 6, after 1
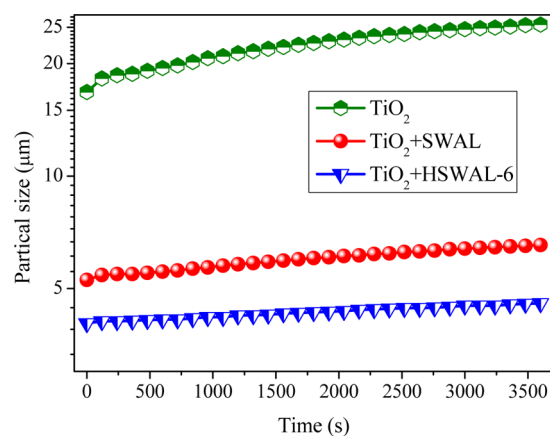

Figure 6. Dispersion performance of SWAL by HRP modification on TiO_2 slurry.

h standing, the diameter of the TiO_2 particles increased to 25 μm. In this study, the influence of HRP modification on the dispersion performance of SWAL on the TiO_2 particles was investigated. As presented in Figure 6, the dispersion performance of HSWAL-6 was obviously better than SWAL. The diameter of the TiO_2 particles was only approximately 4.0 μm after the addition of HSWAL-6. A lower value of particle size indicates a more stable suspension and less agglomerates of TiO_2 slurry. Due to the higher molecular weight and hydrophilic group content, when HSWAL-6 was adsorbed on

the surface of TiO_2 particles, it was beneficial for increasing the steric hindrance and electrostatic repulsion between TiO_2 particles. Therefore, HRP modification could significantly enhance the dispersion performance of sulfomethylated alkali lignin.

CONCLUSIONS

This study demonstrated that HRP could polymerize SWAL efficiently. HRP could oxidize phenolic groups to phenoxyl radicals, undergoing radical–radical couplings. After the HRP modification, the molecular weight, sulfonic, and carboxyl group content increased by 470%, 55%, and 75%, respectively. Moreover, HRP modification significantly improved the adsorption amount of SWAL. When using HSWAL-6 as a dispersant of TiO_2 slurry, the dispersion performance was obviously enhanced. Therefore, the combination of sulfomethylation and HRP modification is a promising method for the more efficient application of alkali lignin.

ASSOCIATED CONTENT

Ⓢ Supporting Information
^{1}H NMR spectra of WAL, SWAL, and HSWAL-6. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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