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<th><strong>Journal:</strong></th>
<th><em>Green Chemistry</em></th>
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<td><strong>Manuscript ID:</strong></td>
<td>GC-ART-05-2015-001083.R1</td>
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<td><strong>Article Type:</strong></td>
<td>Paper</td>
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<td><strong>Date Submitted by the Author:</strong></td>
<td>30-Jul-2015</td>
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<td><strong>Complete List of Authors:</strong></td>
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We report a new assay for breakdown of high molecular weight insoluble lignin based on lignin films. In this method, decrease in film thickness is detected upon solubilization of mass through either chemical alteration of the lignin or molecular weight reduction. The assay was performed with organosolv lignin, the only chemical modification being an oxidative pretreatment to provide film stability with respect to dissolution. The assay is sensitive to release of as little as 20 Å of material from the film. A multiplexed format was developed using a silicone block in the form of a standard 96-well plate, allowing simultaneous assaying of a large number of reaction conditions. The assay was demonstrated using the Fenton reaction, revealing new insights into the physicochemical aspects of this reaction system with insoluble lignin. In particular, mass solubilized from the film was found to pass through a maximum as a function of the initial concentration of FeCl₂ ([FeCl₂]₀), with the maximum occurring at [FeCl₂]₀ = 1 mM for [H₂O₂]₀ = 5%. At that condition, solubilization of mass occurs in two stages. The reaction produces mostly ring-opened products of mass greater than 700 g/mol, along with a minority of low molecular weight aromatics. The new insight from this work is an important step toward optimizing this complex reaction system for effective lignin breakdown.

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Introduction
The valorization of lignin is currently one of the greatest challenges in developing economically viable industries based on lignocellulosic biomass.[1] Lignin is an amorphous polymer that binds cellulose fibrils and hemicellulose into a rigid recalcitrant structure in plant cell walls. Lignin is typically 15-40% of the total dry weight of biomass. While lignin represents the only significant source of renewable aromatics on the planet, currently in the pulp and paper and biofuels industries it is considered a waste stream that is typically burned to generate power and heat. Therefore, lignin represents a significant underutilized source of carbon for conversion to commercial products. Developing cost effective, efficient, and rapid routes to break lignin down into aromatic monomers or other valuable products or precursor chemicals is an intense area of current research.[2-6]

An important bottleneck in converting lignin to valuable products is the efficient breakdown of polymeric lignin into small molecules, suitable for either chemical or biological upgrading.[2-4] The structure and chemistry of lignin varies with the method used for its isolation and separation. Consequently the difficulty of breakdown varies as well. The presence or absence of condensed structures and native ether bonds are factors that most strongly impact the difficulty of further breakdown and conversion. With some lignin breakdown methods, degradation of high molecular weight lignin may be more challenging for water-insoluble forms that arise from most biomass
pretreatment processes in biorefineries and from the kraft and organosolv processes in the pulp and paper industry compared to water-soluble forms such as lignosulfonates.

In nature, microbial communities use both enzymatic and catalytic mechanisms to break down lignin. Microbial degradation of lignin has been well studied in fungi (e.g., white rot and brown rot) and in a small number of bacteria, such as Sphingomonas paucimobilis and Pseudomonas putida, as reviewed elsewhere.[7-11] White rot fungi are able to mineralize lignin using peroxidases and laccases, very likely in conjunction with small molecule mediators. In comparison, brown rot fungi alter the structure of lignin, but do not mineralize it, using Fenton chemistry.[7, 10, 12] Some evidence suggests that white rot fungi also utilize Fenton chemistry to some extent in lignin breakdown.[13-20] The breakdown of lignin in nature by bacteria and fungi is typically too slow for industrial applications. Consequently nonnatural catalytic routes to lignin breakdown are under extensive study.[2, 6, 21]

A major difficulty in the study of lignin breakdown is the lack of a method that is both rapid and highly sensitive for assaying the degradation of polymeric insoluble lignin, since the released fragments are chemically complex and are not easily detected using colorimetric assays. This is in contrast to polysaccharides, in that many methods are available to detect the release of soluble sugars. If the aromatic rings are intact, soluble lignin fragments can be detected through UV absorbance [22, 23] or native fluorescence. However the signals are weak and some extent of ring opening often occurs. A common assay for depolymerization of polymeric lignin for many decades has been the mineralization of C14-labeled dehydrogenase polymeric (DHP) lignin to CO2 [11, 24, 25]. However, DHP lignin is linear and therefore significantly different from the network structure of native lignin. Another approach is to follow changes in the molecular weight distribution of lignin by chromatography.[23, 26-28] This approach is difficult to quantify and also has the disadvantage of requiring dissolution of the sample in an organic solvent or aqueous solution of high alkalinity. Since lignin is held together by both covalent and non covalent interactions [29, 30] changing the solvating conditions can substantially alter the structure, and will not provide insight into the fragments released in near neutral aqueous processing conditions. Importantly, all the above methods require incubating lignin with enzymes or microorganisms for 7-14 days at a minimum to detect significant changes. In addition, it is challenging to see how these approaches could be used to process a large number of samples in a high throughput or highly multiplexed format.

Two new assays involving polymeric lignin have been reported recently with sufficient sensitivity to detect changes within 20 min upon incubation with culture supernatant of lignolytic bacteria and fungi.[24] One approach involves fluorescently-labeled lignin. In this method, breakdown of lignin results in a change in the environment of the fluorophore (FITC) and therefore a change in the fluorescence spectrum. While sensitive to global changes in lignin structure, the release of soluble fragments is not detected. The second method involves chemically nitrated lignin in which 35-45% of the phenolic rings are nitrated. Release of nitrated low molecular weight phenols results in an increase in UV absorbance at 430 nm. The success of this method in rapidly screening and ranking the lignolytic activity of a set of fungi and bacteria represents a substantial improvement over prior methods. However, the method does not quantify the extent of lignin degradation. Furthermore, while the chemically-altered polymeric lignin is closer in structure to native lignin than low molecular weight compounds or DHP, the chemical structure of the lignin is substantially altered. In particular, as a result of nitration the lignin is soluble in aqueous solution in a manner similar to sulfonation, and therefore is not representative of insoluble native lignin or of the insoluble lignin streams in biorefineries.

Despite this progress, there remains a need for a highly sensitive quantitative assay involving polymeric water-insoluble lignin that can be used in a multiplexed format for rapid, high throughput screening of ligninolytic enzymes, microorganisms, and catalytic chemistries. With intensive efforts underway to discover new enzymes and catalysts, along with the need to optimize reaction conditions, there is a need to conduct large-scale screening and to quantify the extent of lignin breakdown. Regarding optimization of conditions for lignin breakdown, both enzymatic and catalytic approaches are plagued by the tendency for
repolymerization of fragments, [30-34] and the physicochemical aspects of this are not fully understood.

We report a new assay based on films of insoluble lignin. In this method, the amount of mass released from the film as soluble fragments is quantified through the decrease in film thickness measured by ellipsometry. The method is quantitative and highly sensitive, as film thickness can be determined to within +/- 20 Å. While the film format precludes interrogation by NMR, chemical bonding within the film can be studied by surface analytical techniques such as FTIR-ATR or XPS, and the chemical nature of the fragments released can be identified by mass spectrometry, FTIR, and UV-vis. The lignin film assay was developed into a multiplexed format using a silicone mold in the form of a standard 96-well plate. When combined with a lignin film on a standard 3-in silicon wafer, the device enables simultaneous assaying of 78 independent reactions.

The results are presented in two sections. First, studies of stability of the lignin films in various conditions are presented. The assay is then demonstrated by quantifying mass release resulting from the Fenton reaction. The Fenton reaction, in which highly potent hydroxyl radical is generated by reaction of hydrogen peroxide and ferrous salt, is used to treat industrial waste and organic contaminants in groundwater,[35-38] and has been employed in biomass pretreatment strategies.[39-41]

Results

A. Stability of the lignin films against dissolution in aqueous solutions

In order to study chemical breakdown of lignin deposited as a film, the film must be stable with respect to dissolution under a variety of conditions. This requires adhesion of lignin to the substrate as well as cohesion among the lignin particles within the film. To provide adhesion to the substrate, thin films of polyethylenimine (PEI) and aminopropyltriethoxysilane (APS) were both examined. Greater adhesion resulted with APS than with PEI. Care was taken to prepare the adhesion-promoting films without substantially increasing surface roughness or lateral variation in film thickness, as each of these limits the accuracy of the lignin film thickness measurement. Details of film preparation are given in the Methods section. As spun, the lignin films experience gradual release of mass upon incubation against water at pH 4.5 and higher when the liquid is strongly agitated. For example, the film thickness decreases by ~ 150 Å when individual film pieces are submerged in 2 ml of liquid in a vial and agitated on a shaker at 60 rpm for 4 hr. Mass release is most likely due to the solubility of low molecular weight and/or more hydrophilic components within the polydisperse sample. To increase film stability with respect to dissolution in conditions that may arise in lignin breakdown studies such as the presence of agitation, high concentrations of H₂O₂, various buffers, and detergents, the lignin films were exposed to solutions of oxidized 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as described in the Methods section. Laccase oxidizes ABTS to the stable cation radicals ABTS⁺ and ABTS²⁺ which oxidize phenolic and nonphenolic structures in lignin, respectively.[42, 43] Stability of the ABTS-treated films was first tested by incubation against 1% Tween 20 for 2 hr in a shaker at 60 rpm. Film thickness as a function of incubation time against oxidized ABTS and also after subsequent exposure to 1% Tween 20 for 2 hrs is shown in Figure 1. Results are shown for films incubated against i) a solution of 25 µg/ml laccase and 1 mM ABTS in 10 mM lactate buffer pH 4.5, ii) a solution of laccase-oxidized ABTS (25 µg/ml laccase and 1 mM ABTS) in 10 mM lactate buffer pH 4.5 incubated for 30 min followed by removal of the laccase...
by filtration), and iii) a solution of 1 mM ABTS in the same buffer (no exposure to laccase). For each of these solutions the film thickness initially decreased by ~150 Å and then levelled off after several hours. The fact that the same amount of mass release occurred for unoxidized ABTS as for oxidized ABTS indicates this is likely due to a low level of solubility along with agitation, rather than reaction of oxidized ABTS with the film. However, resistance to the detergent solution (Tween 20) increased markedly with increase in time exposed to oxidized ABTS, and lignin films incubated for 18 hrs against oxidized ABTS showed no detectable mass release upon incubation with 1% Tween 20 for 2 hr. Physical and chemical changes to the lignin upon treatment with oxidized ABTS are described below. Films treated for 18 hr with oxidized ABTS are hereafter referred to as base films for the lignin breakdown assays.

Stability of the base lignin films was examined further by incubating small wafer pieces against 2 ml of 10 mM sodium lactate buffer pH 4.5, 1% Tween 20, 1% SDS, and 1% TritonX-100 for 18 hrs at room temperature (RT) on a shaker at 60 rpm. No loss of film thickness was detected in the buffer. The results for 18 h incubation in the detergent solutions are shown in Figure 2. Films treated with oxidized ABTS are highly stable against 1% Tween 20 and 1% SDS, but are only partially stable with respect to 1% Triton X-100. Brief incubation in methanol or dioxane completely removed the films, which shows that the films are not crosslinked but are still composed of independent lignin particles or macromolecules. Upon incubation at 90 °C for 16 hr in 50 mM Na malonate buffer pH 4.5 (no agitation) release of ~150 Å was observed. Finally, regarding the effect of pH the base films are stable for several hr in most common buffers at pH 8 absent agitation, but substantial mass loss occurs at longer times or upon agitation. Thus, while exposure to oxidized ABTS increases the stability of the films in aqueous solutions, the films are still susceptible to dissolution in organic solvents, or partial dissolution in alkaline solutions, in buffers at high temperatures, or in certain detergent solutions. Nevertheless, such films are entirely suitable for studies involving enzymes or catalytic reactions in aqueous buffers with neutral or acidic pH and near ambient temperature. We note further that the extent of dissolution is a strong function of the agitation of the liquid. Dissolution is much reduced in 300 µl wells of the multiplexed assay to be described below in absence of shaking compared with the level of dissolution that occurs with 2 ml of liquid in a vial on a shaker at 60 rpm.

**Characterization of films treated with ABTS.**

**MW distribution.** We investigated whether the treatment with laccase-oxidized ABTS alters the molecular weight distribution of the deposited lignin. Crosslinking of lignin by laccase has been reported previously,[44-46] whereas a mixture of laccase and ABTS has been reported to inhibit lignin polymerization.[47] In Figure 3, a GPC trace of the original lignin material is compared with traces for lignin films recovered from wafers that had (+) or were not (-) treated with oxidized ABTS.
not (-) been treated with oxidized ABTS. The lignin films were removed from a large number of 3” diameter silicon wafers using methanol and combined to generate enough material for GPC analysis. The comparison in Figure 3 shows that treatment with oxidized ABTS does not significantly alter the molecular weight distribution.

Film Chemistry. Chemical bonding within the ABTS-treated films was characterized using Fourier transform infrared spectroscopy in attenuated total reflection mode (FTIR-ATR), X-ray photoelectron spectroscopy (XPS), cyclic voltametry, and advancing water contact angles. FTIR spectra were acquired in ATR mode for spin-coated lignin films both before and after treatment with oxidized ABTS. The spectrum for a thick (essentially bulk) film prepared by allowing droplets of 3% lignin in dioxane to evaporate on a Teflon substrate is compared with the spectra of a base lignin film (~ 900 Å) before and after treatment with oxidized ABTS in Figure 4. Absorption bands were assigned following prior studies [48-54]: 1706 cm\(^{-1}\) C=O stretch in unconjugated ketones, carbonyls, and ester groups with conjugated carbonyls and carboxyls slightly lower toward the 1650 cm\(^{-1}\) range; 1598 cm\(^{-1}\), 1514 cm\(^{-1}\), and 1422 cm\(^{-1}\) aromatic skeletal vibrations; 1456 cm\(^{-1}\) C-H deformation combined with aromatic ring skeletal vibration; 1325 cm\(^{-1}\) S ring plus G ring condensed; 1214 cm\(^{-1}\) C-O stretch (aryl C-O); 1117 cm\(^{-1}\) C-O stretch (ethers) potentially superposed with aromatic C-H in-plane deformation; 1083 cm\(^{-1}\) C-H deformation in secondary alcohols and aliphatic ethers; 1031 cm\(^{-1}\) aromatic C-H in-plane deformation and C-O stretch (alcohols); and 869 cm\(^{-1}\) and 833 cm\(^{-1}\) aromatic C-H out of plane deformation. The relation of the band at 1456 cm\(^{-1}\) to aromaticity is somewhat ambiguous, since the C-H deformation of CH\(_3\) groups including methoxy occurs in this region as does a strong aromatic ring vibration. The C-O stretch bands are usually observed in the region from 1240-1030 cm\(^{-1}\), and are expected to be in the order of aryl C–O > ether > alcohol and were assigned accordingly. For this region some discrepancy exists in the literature, as a C=O contribution to bands in this region has been suggested [50, 52, 54]. However, carbonyls are not expected to adsorb in this region.[48] Comparing the spectra for the 900 Å spin-coated film to that of the much thicker film, the absorbance at 1214 cm\(^{-1}\) for the thick film has been replaced by a doublet at 1200 cm\(^{-1}\). Following others,[48, 50, 54] we assign these absorbances to C-O stretch of aryl C-O. In addition, the ratio of the bands at 1117 cm\(^{-1}\) and 1080 cm\(^{-1}\) is altered in the spin-coated 900 Å film such that the band at 1080 cm\(^{-1}\) is stronger.
changes occur upon treatment with oxidized ABTS, indicating that chemical modification of the lignin films upon treatment with oxidized ABTS is relatively weak and not easily detectable by FTIR-ATR.

XPS analysis indicated an increase in oxygen content from 22.3% to 26.1% (O 1s) and a decrease in carbon content from 77.3% to 73.4% (C 1s) upon ABTS treatment. Nitrogen content was less than 0.4% (N 1s).

Cyclic voltammetry was performed on a lignin film after treatment with laccase-oxidized ABTS. A prior study of a lignin film reported a peak corresponding to oxidation of phenolic groups in the initial scan that was replaced by a peak corresponding to oxidation of quinones in subsequent scans.[55] Our results in Figure 5 show only a reversible quinone redox peak but no phenol peak. This suggests that phenolic groups are oxidized to quinones by the ABTS treatment. Oxidation of phenolic compounds to quinones has been reported previously.[56]

Advancing water contact angles on the base films were time dependent, indicating that surface reconstruction occurred. Values typically decreased from 70° to 63° during a 5 minute period. This further shows that the films are not highly crosslinked, but rather contain particles or macromolecules that are able to move independently and rearrange.

In summary, treatment of the lignin films by laccase-oxidized ABTS results in a 3-4% increase in oxygen content, oxidation of phenolic groups to quinones, and no detectable change in the molecular weight distribution.

B. Lignin breakdown by the Fenton reaction

The assay was initially performed using individual wafer pieces (~5 mm x 5 mm) with base films and solutions of [H$_2$O$_2$]$_o$ = 3% with [FeCl$_3$]$_o$ of 1 mM and 4 mM at room temperature (RT). The pH was constant throughout the reaction at 3.2 and 2.9 for [FeCl$_3$]$_o$ of 1 mM and 4 mM, respectively. Results are shown in Figure 6. Mass release occurred in stages, with a higher rate of mass release within the first 3 hours followed by a lower rate of mass release. The initial rate of mass release was lower for [FeCl$_3$]$_o$ = 1 mM than for [FeCl$_3$]$_o$ = 4 mM, but midway through the incubation period the rate of mass release for [FeCl$_3$]$_o$ = 1 mM increased substantially such that at the end of 18 h substantially greater thickness decrease occurred for [FeCl$_3$]$_o$ = 1 mM than for [FeCl$_3$]$_o$ = 4 mM. Incubation in a solution of [H$_2$O$_2$]$_o$ = 3% (no FeCl$_3$) resulted in mass release from the films at a much lower level than for the solutions containing FeCl$_3$. Figure S1 and Figure S2 in the Supporting Information provide further results for base films incubated in H$_2$O$_2$ solutions under different conditions. As discussed further below, mass release in H$_2$O$_2$ solutions is most likely due to a slight solubility of the lignin particles at high concentrations of H$_2$O$_2$. For the case of [FeCl$_3$]$_o$ = 4 mM and [H$_2$O$_2$]$_o$ = 3% incubated for 18 h, a second treatment resulted in further mass release of comparable magnitude as for the original treatment.
The lignin film assay was subsequently adapted to a multiplexed format using a silicone rubber block containing an array of holes in the pattern of a standard 96-well plate (Fig S3). This allows 78 reaction conditions to be assayed simultaneously with a standard 3" in silicon wafer. Figure 7a shows a photograph of a lignin film-coated silicon wafer following incubation for 18 hr against solutions containing [H\(_2\)O\(_2\)]\(_o\) of 3% and 5% with [FeCl\(_2\)]\(_o\), ranging from 0 mM to 4 mM. The pH ranged from 2.96 to 2.83 for [H\(_2\)O\(_2\)]\(_o\) = 3%, and from 2.82 to 2.72 for [H\(_2\)O\(_2\)]\(_o\) = 5%. The decrease in film thickness at the location of each well is plotted in Figure 7b. Rows 1–4 were identical and rows 4–8 were identical in order to assess reproducibility. The error bars in Figure 7b are the standard deviation of the four replicates for each condition. The plot shows that mass loss from the film is a nonlinear function of [FeCl\(_2\)]\(_o\). In particular, there is a sharp increase in mass release over a narrow range of [FeCl\(_2\)]\(_o\), followed by a slow decline from the maximum with further increase in [FeCl\(_2\)]\(_o\). The transition to the maximum mass release with [FeCl\(_2\)]\(_o\) is sharper for [H\(_2\)O\(_2\)]\(_o\) = 5% than for [H\(_2\)O\(_2\)]\(_o\) = 3%. Since the initial thickness of the lignin film was about 900 Å, the greatest mass loss corresponds to release of roughly 2/3 of the film. The results also show that only a very low amount of mass is released for [H\(_2\)O\(_2\)]\(_o\) of 3% and 5% in absence of FeCl\(_2\). The amount of mass solublized from the film in the presence of FeCl\(_2\) increases substantially with [H\(_2\)O\(_2\)]\(_o\). Additional
studies indicated that very little mass release occurs for 
\([\text{H}_2\text{O}]_o \leq 1\% \) over the same range of \([\text{FeCl}_2]_o\).

The multiplexed format was also used to study the
time dependence of mass release for \([\text{H}_2\text{O}]_o = 5\% \) over
the same range of \([\text{FeCl}_2]_o\), (Figure 8). Consistent with
the data in Figure 6 for individual lignin-coated wafer
pieces, Figure 8a shows that very little mass relea
ses for \([\text{FeCl}_2]_o < 0.5 \text{ mM} \), a large increase in mass
release occurs for \([\text{FeCl}_2]_o = 1.0 \text{ mM} \), and mass release after 13 h is substantially greater
for \([\text{FeCl}_2]_o = 4.0 \text{ mM} \) and than for \([\text{FeCl}_2]_o = 1.0 \text{ mM}\).  

Regarding the time course, at \([\text{FeCl}_2]_o = 1.0 \text{ mM} \) there is an initial rapid loss of \(\sim 130 \text{ Å} \) from the film
followed by several hours during which little mass
release occurs. Then between 5 h and 10 h mass release
occurs at a substantially increased rate. Beyond 10 h
the rate of mass release begins to taper off again. For
\([\text{FeCl}_2]_o = 2 \text{ mM} \) and \([\text{FeCl}_2]_o = 4 \text{ mM} \), the shape of the
curve is distinctly different from that at \([\text{FeCl}_2]_o = 1 \text{ mM} \), as shown in Figure 8b. Over the first 5 h the mass
release is greater for \([\text{FeCl}_2]_o = 2 \text{ mM} \) and \([\text{FeCl}_2]_o = 4 \text{ mM} \) than for \([\text{FeCl}_2]_o = 1 \text{ mM} \), but the rate of mass
release tapers off after that, in contrast to the strong
increase in the rate of mass release between 5 h and 10 h
for \([\text{FeCl}_2]_o = 1 \text{ mM} \).  

**FTIR Analysis of Residual Film**. FTIR spectra were
collected from several locations on the lignin film
sample shown in Figure 7a. The locations scanned
correspond to \([\text{FeCl}_2]_o = 1 \text{ mM} \), \(1.5 \text{ mM} \), and \(4 \text{ mM} \) for
\([\text{H}_2\text{O}]_o \) of 3% and 5%. The spectra in the region from 1150 cm\(^{-1}\) to 1800 cm\(^{-1}\) are compared in Figure 9. The
spectra corresponding to \([\text{FeCl}_2]_o = 1 \text{ mM} \) (reaction
conditions giving the maximum mass loss) show
substantial changes compared with the spectrum for the
original lignin film whereas the spectra for \([\text{FeCl}_2]_o = 4 \text{ mM} \) are similar to that of the original film. While the
post-reaction lignin film is substantially thinner for
\([\text{FeCl}_2]_o = 1 \text{ mM} \), the spectra are not simply reduced in
magnitude but show distinct changes in the vibrational
bands corresponding to chemical differences in the film.
In particular, the aromatic bands and the band at 1700
cm\(^{-1}\) are greatly diminished and a broad absorbance is
observed from 1500 cm\(^{-1}\) to 1650 cm\(^{-1}\). These two trends
suggest that substantial alteration of the aromatic
structures occurred. We propose that opening of
aromatic rings leads to conjugated systems of aliphatic C=C and carboxylate (C=O) groups that absorb in the
region from 1550 cm\(^{-1}\) to 1650 cm\(^{-1}\). Loss of the C=O
stretching band at 1700 cm\(^{-1}\) is easily explained by a
shift to lower wavenumber when acids, aldehydes, and
ketones are conjugated with C=C bonds. The spectra
corresponding to the intermediate value of \([\text{FeCl}_2]_o =
spectrum at \([\text{FeCl}_2]_0 = 1.5 \text{ mM}\) is intermediate between that at \([\text{FeCl}_2]_0 = 1.0 \text{ mM}\) and \([\text{FeCl}_2]_0 = 4.0 \text{ mM}\), showing a slight decrease in the aromatic peaks and a slight broadening of absorbance from 1500 cm\(^{-1}\) to 1650 cm\(^{-1}\).

FTIR spectra obtained for base films before and after 18 h incubation with 3\% \(\text{H}_2\text{O}_2\) (i.e. no \(\text{FeCl}_2\)) show no significant changes (Figure S4), supporting the conclusion that the low level of mass release in that case is due to solubilization by virtue of \(\text{H}_2\text{O}_2\) rather than chemical reaction.

**UV absorbance of solubilized products.** UV absorbance spectra of liquids from the wells of the film assay after reaction for \([\text{FeCl}_2]_0 = 1.0 \text{ mM}\) and \([\text{FeCl}_2]_0 = 4.0 \text{ mM}\) at \([\text{H}_2\text{O}_2]_0 = 5\%\) are compared in Figure 10, along with the spectrum for the water-soluble fraction of the original lignin material. Bands at 205 nm and 280 nm characteristic of lignin are present in the spectrum of the reaction liquid for \([\text{FeCl}_2]_0 = 4.0 \text{ mM}\) but are absent in the spectrum for \([\text{FeCl}_2]_0 = 1.0 \text{ mM}\). This indicates that substantially more aromaticity is present in the solubilized products from the reaction involving \([\text{FeCl}_2]_0 = 4.0 \text{ mM}\) than for \([\text{FeCl}_2]_0 = 1.0 \text{ mM}\).

**FTIR of solubilized products.** To analyze the soluble products by FTIR-ATR the liquid from certain wells was removed and evaporated onto Teflon substrates. The material remaining after evaporation was then scraped from the Teflon substrate and pressed against the ATR diamond probe. In Figure 11a the FTIR absorbance spectrum for the wells corresponding to the last three time points for \([\text{FeCl}_2]_0 = 1 \text{ mM}\) in Figure 8 is compared to the spectrum for the well corresponding to the 11 h time point for \([\text{FeCl}_2]_0 = 4 \text{ mM}\) in Figure 8. These times correspond to the second phase of mass loss, which is strong for \([\text{FeCl}_2]_0 = 1 \text{ mM}\) but not observed for \([\text{FeCl}_2]_0 = 4 \text{ mM}\). Upon evaporation the material for \([\text{FeCl}_2]_0 = 4 \text{ mM}\) formed a thin film on the Teflon substrate whereas the material for \([\text{FeCl}_2]_0 = 1 \text{ mM}\) formed a particle rather than a film. This indicates a substantial difference in the nature of the material for the two conditions. The difference in behaviour upon evaporation could be due to a difference in molecular weight distribution or to a difference in the chemical nature of the fragments. The difference is most likely due to a difference in molecular weight, as the IR spectra show the same three major bands in the

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**Figure 10.** UV absorbance spectra from reaction liquids for \([\text{FeCl}_2]_0 = 1.0 \text{ mM}\) and \([\text{FeCl}_2]_0 = 4.0 \text{ mM}\) at \([\text{H}_2\text{O}_2]_0 = 5\%\), indicating substantial chemical differences in the solubilized products. The inset shows the spectrum for the original lignin material (water-soluble fraction).

**Figure 11.** a) FTIR spectra from material remaining after evaporation of the liquid from wells corresponding to the last three time points for \([\text{FeCl}_2]_0 = 1 \text{ mM}\) in Figure 8 (red) and from the well corresponding to the 11 h time point for \([\text{FeCl}_2]_0 = 4 \text{ mM}\) in Figure 8 (blue). b) Expanded view of the spectrum for \([\text{FeCl}_2]_0 = 4 \text{ mM}\) in a) along with the spectrum for the lignin film prior to reaction.
fingerprint region for [FeCl$_3$]$_o$ = 1 mM and [FeCl$_2$]$_o$ = 4 mM. Importantly, the spectra show no evidence for aromatics at either reaction condition. The definitive band for aromatics is 1510-1515 cm$^{-1}$. The absence of this band for both [FeCl$_3$]$_o$ = 1 mM and [FeCl$_2$]$_o$ = 4 mM indicates that aromatics are present in low abundance in both cases, despite the presence of a band at 280 nm characteristic of aromatics in the UV spectrum (Fig 10) for [FeCl$_3$]$_o$ = 4 mM. We conclude that the soluble material is predominantly comprised of ring-opened products in both cases, but that a small minority of aromatics are present in the solubilized material for [FeCl$_3$]$_o$ = 4 that are detected in the UV but not in the IR due to the greater sensitivity of UV absorbance to aromatics. While the soluble material is predominantly comprised of ring-opened products in both cases, the distribution of ring-opened products appears to differ somewhat. The absorbance band from 1635 cm$^{-1}$ to 1725 cm$^{-1}$, corresponding to carbonyl and carboxyl groups, and the band at 1017 cm$^{-1}$ which we assign to the C-O stretch in ethers and alcohols, are much stronger at [FeCl$_3$]$_o$ = 1 mM than at [FeCl$_2$]$_o$ = 4 mM relative the broad OH band at 3000-3400 cm$^{-1}$. We tentatively interpret this as indicating a distribution of products with greater tendency to bind water strongly in the case of [FeCl$_3$]$_o$ = 4 mM. In addition, the ratios of the two major bands in the fingerprint region (1635 cm$^{-1}$ - 1725 cm$^{-1}$ and the band at 1017 cm$^{-1}$) to that at 1420 cm$^{-1}$, which we assign to C-H stretch, are greater at [FeCl$_3$]$_o$ = 1 mM than at [FeCl$_2$]$_o$ = 4 mM. Further work is required to resolve the detailed differences in the distribution of ring-opened products for the two cases.

LC-MS Analysis of low MW soluble products
Results from LC-MS analyses of solubilized products from the Fenton reaction are given in the supporting information (Figs S5-S8). LC protocols for low MW aliphatic acids, aromatics by atmospheric pressure chemical ionization (APCI), and aromatic acids and aldehydes by electrospray ionization (ESI) were performed. The MS analyses covered molecular weights from 100 g/mol to 700 g/mol. Regarding low MW acids, oxalate is present in far greater amounts than either malonate, succinate, or glycolate. Oxalate is present in much greater abundance at [FeCl$_3$]$_o$ = 4 mM than at [FeCl$_2$]$_o$ = 1 mM. This is not unexpected as oxalic acid is generated in the oxidation pathway of the other acids, and is the last species generated prior to mineralization.[57] Regarding aromatics by APCI, mass 218.17 is present in far greater amounts than all other masses. This species is present in comparable amounts at [FeCl$_3$]$_o$ = 1 mM and at [FeCl$_2$]$_o$ = 4 mM. Regarding aromatic acids and aldehydes by ESI, masses 195.09, 368.27, and 424.33 g/mol are present in greatest abundance. These species are also present in comparable amounts at [FeCl$_3$]$_o$ = 1 mM and at [FeCl$_2$]$_o$ = 4 mM. Importantly, while these data identify the most prominent fragments released in the mass range from 100 – 700 g/mol, none of the most abundant species over this mass range is in large excess at [FeCl$_3$]$_o$ = 1 mM compared with [FeCl$_2$]$_o$ = 4 mM.

Discussion

The assay described here employs high MW insoluble lignin rather than low MW or soluble forms of lignin as in most other assays of lignin degradation. Moreover, the assay can be performed in aqueous buffers at acidic or near neutral pH, without the need to employ organic solvents or highly alkaline solutions that can alter the physical state of the lignin. The assay is highly sensitive (detects release of as little as 20 Å from the film), potentially enabling detection of ligninolytic activity after short incubation times. While quantitative changes in film thickness from ellipsometric measurements were reported above, changes in film thickness are qualitatively apparent from changes in color. Since the films are ultrasmooth and highly uniform, loss of as little as 30 Å from the films leads to a change in color that is visually observable. An example of the variation of film color with thickness is shown in Fig 12. This allows for very rapid qualitative assessment of the extent of degradation. Development of the lignin film assay into a multiplexed format allows a large number of reaction conditions to be assayed simultaneously. In this work, organosolv lignin [58] was used as the substrate, however the assay method is suitable for use with other types of lignin. Organosolv lignin is known to be somewhat recalcitrant to further depolymerization, in
that it is extensively depolymerized through cleavage of ether bonds during the organosolv process.[59, 60] In this work, the lignin films were stabilized against dissolution using laccase-oxidized ABTS. Such films are suitable for use with high concentrations of H2O2, neutral or slightly basic conditions, mild agitation, or with lignolytic microbes where rinsing with detergent solutions is required. However, stabilization by treatment with oxidized ABTS is not required for studies of enzymes in buffers at near-neutral or acidic pH in the multiplexed format, as film dissolution is minimal within the 300 µl wells for such conditions in absence of mixing/shaking. Application of this assay at higher temperatures, high pH values, or mixed solvent (aqueous + organic) systems will require more effective methods of stabilizing the lignin particles within the film with respect to dissolution.

**Fenton reaction.** In general terms, ‘Fenton chemistry’ can be described as oxidation based on activated hydrogen peroxide, where a redox cycle of Fe(II) and Fe(III) facilitates free radical oxidation with hydrogen peroxide being the primary oxidant. Fe(II) containing oxidizing environments facilitate hydrogen peroxide decomposition to yield hydroxyl and hydroperoxyl radicals as strongly propagating intermediates. Fenton approaches have been used to facilitate degradation of polymers in aqueous and nonaqueous solutions.[61, 62] The Fenton reaction system has also been extensively studied for degradation of dilute water-soluble small molecule (mostly aromatic) substrates for water purification. Following Kang et al.[63] the primary reactions in absence of a substrate are given in Table 1. The reactions of this system with certain soluble aromatic compounds are also fairly well understood. For these homogenous reaction conditions, good understanding exists including rate constants for the primary reactions, such that changes in reactant and product concentrations with time have been modelled with reasonable accuracy.[63-68] However, heterogeneous reaction of the Fenton system with insoluble polymeric lignin is much more complex.[69] Lignin fragments generated by reaction with hydroxyl radicals can either recombine with the lignin mass or can diffuse away into solution. This is controlled primarily by the solubility of the fragments, but may also be affected by the local density of the lignin (highly condensed or more diffuse). Smaller, more highly oxidized fragments have greater solubility in aqueous solutions and low molecular weight acids are the most commonly observed soluble products when the reactants are present at high concentrations.[40] However, at intermediate oxidation levels, reactions leading to ring opening that generate di-acids can render high MW fragments soluble. Another complicating factor, discussed further below, is that functionalities within partially oxidized lignin can bind Fe(III) and facilitate its reduction to Fe(II).[70]

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( k \left( M^{-1} \cdot s^{-1} \right) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R1) Fe^{II} + H2O2 → Fe^{III} + OH + H2O</td>
<td>63</td>
</tr>
<tr>
<td>(R2) Fe^{III} + H2O2 → Fe^{IV} + H^+ + H2O2</td>
<td>0.01</td>
</tr>
<tr>
<td>(R3) OH + H2O2 → HO2^- + H2O</td>
<td>2.7 x 10^7</td>
</tr>
<tr>
<td>(R4) HO2^- → O2^- + H^+</td>
<td>1.58 x 10^7 s^-1</td>
</tr>
<tr>
<td>(R5) O2^- + H^+ → HO2^-</td>
<td>1.0 x 10^6</td>
</tr>
<tr>
<td>(R6) OH + Fe^{IV} → Fe^{II} + OH^-</td>
<td>3.2 x 10^8</td>
</tr>
<tr>
<td>(R7) HO2^- + Fe^{II} (H^+) → Fe^{III} + H2O2</td>
<td>1.2 x 10^6</td>
</tr>
<tr>
<td>(R8) HO2^- + Fe^{III} → Fe^{IV} + H^+ + O2</td>
<td>3.1 x 10^7</td>
</tr>
<tr>
<td>(R9) O2^- + Fe^{IV} (12H^+) → Fe^{III} + H2O2</td>
<td>1.0 x 10^7</td>
</tr>
<tr>
<td>(R10) O2^- + Fe^{III} → Fe^{II} + O2</td>
<td>5 x 10^5</td>
</tr>
<tr>
<td>(R11) OH + OH^- → H2O2</td>
<td>4.2 x 10^9</td>
</tr>
<tr>
<td>(R12) HO2^- + HO2^- → H2O2 + O2</td>
<td>8.3 x 10^5</td>
</tr>
<tr>
<td>(R13) OH + HO2^- → O2 + H2O</td>
<td>1 x 10^19</td>
</tr>
<tr>
<td>(R14) OH + O2^- → O2 + OH^-</td>
<td>1 x 10^19</td>
</tr>
<tr>
<td>(R15) HO2^- + O2^- (OH^-) → H2O2 + O2</td>
<td>9.7 x 10^7</td>
</tr>
</tbody>
</table>

The reaction pathways of hydroxyl radical with soluble aromatic compounds have been studied in great detail. It was shown previously that hydroxyl radical reacts with aromatic compounds mainly by addition to the ring rather than by reaction with substituents on the ring.[71-75] resulting in hydroxycyclohexadienyl radicals that react further by different pathways depending on reaction conditions such as pH and the presence of O2, oxidants, or reductants.[64, 71, 76, 77] These different pathways lead to either loss of aromaticity in the form of cyclohexadienyl species or ring-opened products, or various products in which the aromatic rings are retained. The latter includes condensation reactions of two rings and also formation of various phenols. For interaction of hydroxyl radical with lignin, the same reaction pathways have been reported, along with displacement of methoxy groups and cleavage of β-O-4 ether bonds.[14, 70, 78, 79]
Understanding the factors that control these reaction pathways in the context of the heterogeneous reaction with insoluble lignin will result in the potential for reaction optimization, including control over the product distribution and the yield of soluble products, and more efficient use of the expensive reagent \( \text{H}_2\text{O}_2 \).

The data in Figures 6-8 for the heterogeneous reaction of the Fenton system with lignin films show several features that contrast with the results of prior studies involving homogenous reactions with dilute soluble aromatic substrates. First, much higher \( \text{H}_2\text{O}_2 \) is needed to achieve significant mass release from lignin films compared with that required to degrade dilute soluble substrates. Specifically, Figure 7 shows that mass release from the lignin films during an 18 h incubation is substantially lower for \( \text{H}_2\text{O}_2 \leq 5\% \) compared with \( \text{H}_2\text{O}_2 = 5\% \), and further studies (not shown) indicate that very little mass release is observed for \( \text{H}_2\text{O}_2 \leq 1\% \) (= 294 mM). In contrast, \( \text{H}_2\text{O}_2 = 10-20 \text{mM} \) is adequate to degrade dilute soluble substrates for \( \text{FeCl}_2 \) in the mM range. [63-68] Second, at \( \text{FeCl}_2 = 1 \text{mM} \) mass release occurs in two stages (Figure 8). In contrast, the degradation of dilute soluble aromatic substrates typically occurs within a few tens of minutes at mM concentrations of \( \text{FeCl}_2 \) and \( \text{H}_2\text{O}_2 \) and there appears to be only a single stage in the reaction. [63-65]

Third, the amount of mass solubilized from the lignin film during an 18 h incubation goes through a maximum as a function of \( \text{FeCl}_2 \), whereas the rate of degradation of soluble aromatic substrates increases monotonically with \( \text{FeCl}_2 \). [63-65] Each of these aspects will be discussed further below.

Prior to discussing possible mechanisms to account for these observations, we first summarize the analytical results for the soluble products. The IR data for the soluble products indicates that the main components are carboxylic acids, aldehydes, ketones, ethers, and alcohols, and the lack of detectable aromatic bands indicates that aromatics are present in small amounts for both \( \text{FeCl}_2 = 1 \text{mM} \) and \( \text{FeCl}_2 = 4 \text{mM} \). With UV absorbance, which is more sensitive to aromatics than IR, aromatics are detected at \( \text{FeCl}_2 = 4 \text{mM} \) but not at \( \text{FeCl}_2 = 1 \text{mM} \). LC-MS has the greatest sensitivity of the three methods, but only covered the mass range from 100-700 g/mol. Aromatics were detected at \( \text{FeCl}_2 = 1 \text{mM} \) and at \( \text{FeCl}_2 = 4 \text{mM} \), and the most prominent species were present at roughly the same abundance in the two cases. From this it follows that the aromatic signal detected by UV absorbance for \( \text{FeCl}_2 = 4 \text{mM} \) but absent for \( \text{FeCl}_2 = 1 \text{mM} \) must arise from molecular weights greater than 700 g/mol. Furthermore, the fact that no species was present in substantially greater abundance at \( \text{FeCl}_2 = 1 \text{mM} \) than at \( \text{FeCl}_2 = 4 \text{mM} \) in the MS spectrum indicates that the solubilized species corresponding to the greater mass loss at \( \text{FeCl}_2 = 1 \text{mM} \) (four fold excess at \( \text{H}_2\text{O}_2 \leq 1\% \) relative to that at \( \text{FeCl}_2 = 4 \text{mM} \)) must also have molecular weights greater than 700 g/mol.

Regarding the physicochemical mechanisms of the Fenton system with insoluble lignin, the requirement for higher \( \text{H}_2\text{O}_2 \) for mass loss from insoluble lignin films may simply reflect the fact that multiple oxidation reactions are needed to generate fragments that are sufficiently soluble to diffuse into solution rather than remain with the lignin film. This is especially true for solubilization of higher MW fragments. Another possible explanation is enhanced coupling between hydroxycyclohexadienyl radicals. Prior studies of the reaction of hydroxyl radical with dilute soluble (low MW) aromatics have shown that coupling of two hydroxycyclohexadienyl radicals is favored in the absence of \( \text{O}_2 \) or oxidants. [64, 73, 77] Coupling of two hydroxycyclohexadienyl radicals is likely to be more strongly favored when localized within insoluble lignin particles compared to freely diffusing soluble substrates. Such coupling reactions would not result in the release of mass from the film. For the heterogeneous Fenton reaction involving a film of insoluble lignin particles a much higher concentration of oxidant may be required to bias the reaction away from biphenyl coupling reactions. Third, a higher \( \text{H}_2\text{O}_2 \), may provide a more solubilizing environment for fragments generated from the reaction.

Mass release from the film passes through a maximum because the second stage of mass release is much stronger at \( \text{FeCl}_2 = 1 \text{mM} \) than at \( \text{FeCl}_2 = 4 \text{mM} \). We emphasize the remarkable finding that greater mass release from the film occurs at lower oxidizing power over this range. Regarding the two stages of mass release we propose that the first stage is due to hydroxyl radical generated by reaction of the initial concentration of Fe(II) with \( \text{H}_2\text{O}_2 \) as in eq R1 (Table 1). We propose that the second stage of mass loss occurs upon gradual reduction of Fe(III) to Fe(II), thereby
generating additional hydroxyl radical. This is supported by the fact that the second phase of mass loss occurs at reaction times between 5 and 10 hours, which are much longer than required for complete reaction of the initial concentration of Fe(II).[63-66] Reduction of Fe(III) to Fe(II) is likely to result from the interaction of Fe(III) with dihydroxy aromatics or acids within the lignin.[70] A number of studies have shown that various ortho dihydroxy aromatic compounds and acids are able to reduce Fe(III) to Fe(II).[17, 80-82] The interaction of hydroxyl radical with lignin is known to result in replacement of methoxy groups with hydroxyls, which would leave an abundance of ortho dihydroxy aromatic moieties.[14] We propose that either acid or dihydroxy aromatic functionalities bind and reduce Fe(III) to Fe(II) producing the second stage of mass release for \([\text{FeCl}_2]_o = 1 \text{ mM}\).

In addition to explaining the origin of the second stage of mass release this mechanism also explains why the second state is much more efficient at solubilizing mass from the film than the first stage. During the first stage the amount of mass solubilized is greater at \([\text{FeCl}_2]_o = 4 \text{ mM}\) than at \([\text{FeCl}_2]_o = 1 \text{ mM}\), as expected based on the higher concentration of hydroxyl radical. However, the second stage is much more efficient at releasing mass from the film, resulting in much greater total mass release at \([\text{FeCl}_2]_o = 1 \text{ mM}\) than at \([\text{FeCl}_2]_o = 4 \text{ mM}\). If, as postulated here, the second stage is due to Fe(III) chelation and reduction by dihydroxy aromatic moieties within the lignin, then in that case hydroxyl radical will be generated in close proximity to the aromatic rings. We postulate that this leads to ring opening and generation of carboxylic acid groups that solubilizes high molecular weight fragments in a manner that is very efficient with respect to usage of hydroxyl radical. This is supported by the FTIR and UV absorbance data showing that greater ring opening occurs at \([\text{FeCl}_2]_o = 1 \text{ mM}\) than at \([\text{FeCl}_2]_o = 4 \text{ mM}\).

If, as hypothesized here, the second stage of degradation is due to reduction of Fe(III) to Fe(II), then it follows that at \([\text{FeCl}_2]_o = 4 \text{ mM}\) the reduction of Fe(III) to Fe(II) is suppressed. One possibility is that reduction of Fe(III) to Fe(II) is suppressed at \([\text{FeCl}_2]_o = 4 \text{ mM}\) due to much higher oxalic acid concentration generated in that case (supporting information, Fig S5). Decreased capacity of organic compounds to reduce Fe(III) to Fe(II) is known to occur with increasing oxalic acid concentration due to the formation of highly stable Fe-oxalate complexes.[83] In particular, prior work has shown that excess oxalic acid inhibits Fe(III) reduction by dihydroxy aromatic compounds.[17, 81] Further work is needed to confirm this hypothesis in the present case.

The possibility of different degradation pathways at \([\text{FeCl}_2]_o = 1 \text{ mM}\) and \([\text{FeCl}_2]_o = 4 \text{ mM}\) is suggested by prior work showing that the decay of hydroxycyclohexadienyl radicals to yield ring fragmentation products becomes more significant with increasing concentration of \(\text{O}_2\), whereas the formation of phenols is favored in the presence of a strong oxidant.[64, 71, 77] The UV data showing detectable aromatics at \([\text{FeCl}_2]_o = 4 \text{ mM}\) but no detectable aromatics at \([\text{FeCl}_2]_o = 1 \text{ mM}\) suggests that different decay pathways may be involved. However, the IR data in Figure 11 shows that in both cases the majority of solubilized products are ring-opened species. The fact that the majority of solubilized products are the same in the two cases suggests that any difference in chemical degradation pathways is a minor effect. We suggest that the greater amount of ring opening at \([\text{FeCl}_2]_o = 1 \text{ mM}\) shown in Figure 9 and Figure 10 is the result of oxidation of rings of aromatic moieties that chelated and reduced Fe(III) to Fe(II) during the second phase. This mechanism would open the rings of soluble aromatics produced during the first stage and would also release additional high molecular weight ring-opened products.

Conclusion

With a high sensitivity and multiplexed format, this new assay should be useful for rapid assessment of catalytic, enzymatic, and microbial degradation of insoluble lignin. Furthermore, it can provide new fundamental insights into physicochemical processes involved in lignin degradation, as demonstrated here in the discovery of the nonlinear dependence of mass solubilization on \([\text{FeCl}_2]_o\) for the Fenton reaction system. Time course studies showed that the increased solubilization of mass at \([\text{FeCl}_2]_o = 1 \text{ mM}\) corresponds to a second phase that is not present at \([\text{FeCl}_2]_o = 4 \text{ mM}\). We attribute this second phase of mass release to the gradual reduction of Fe(III) to Fe(II) by dihydroxy
aromatic moieties or aromatic acids generated within the lignin. We suggest that reduction of Fe(III) to Fe(II) is suppressed at [FeCl₂]₀ = 4 mM due to generation of a higher concentration of oxalic acid in that case. However, other possible interpretations may exist and further work is needed to definitely resolve the mechanisms leading to the nonlinear behavior with [FeCl₂]₀. The reaction produces mostly ring-opened products along with a small minority of aromatics. The yield of aromatics is lower at [FeCl₂]₀ = 1 mM than at [FeCl₂]₀ = 4 mM. Taken together the data suggest that the majority of solubilized fragments have molecular weights greater than 700 g/mol. This work shows that a thorough understanding of this complex heterogeneous reaction system is necessary in order to optimize it for degrading lignin.

Experimental

Materials

Organosolv lignin was obtained from Lignol Corp. This material has a bimodal molecular weight distribution. The larger peak of the two peaks is the lower molecular weight fraction, which has Mn = 4250 g/mol, Mw = 9700 g/mol by GPC based on calibration with polystyrene standards. Aminopropyltriethoxysilane (APS), polyethyleneimine (PEI, MW = 60,000 g/mol), FeCl₂, Triton X-100, and 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) were purchased from Sigma-Aldrich. H₂O₂ (~35%) and sodium dodecylsulfate were purchased from Fisher Scientific. Tween 20 was purchased from Fisher Biotech. Laccase from T. versicolor (10 U/mg) was purchased from US Biological. Silicon wafers were undoped type N with 110 orientation.

Methods

Preparation of lignin films. Prior to coating the silicon substrates with lignin, films of either PEI or APS were deposited to promote adhesion. Silicon wafers (3-in. diameter) were first cleaned with detergent solution followed by UV-ozone treatment for 30 min. PEI was deposited by dip coating following a prior report.[84] The wafers were placed into 0.5% solution of PEI in water for 10 min, then removed and rinsed with water, and dried with nitrogen. Smooth films of APS were deposited onto UV-ozone cleaned silicon wafers by spin coating. APS was dissolved into 90/10 ethanol/water at 0.25 % and stirred for 60 min. The solution was then spin coated onto the silicon wafers at 3000 rpm. The APS-coated wafers were then heated in vacuum at 70 C for 1 hr to drive off the water and cure the film.

Lignin was dissolved at 2% or 3% in 1,4 dioxane, filtered through a 1 µM syringe filter, and spin coated onto silicon wafers using a Headway photo resist spinner model 1-PM101DT-R790.[34, 85] A range of spinning speeds (2000 – 4000 rpm) and accelerations (2 – 9) were explored to maximize film uniformity. The lignin-coated wafers were then heated in vacuum at 70 C for 1 hr to drive off the dioxane. At 4000 rpm and acceleration of 9.0, film thicknesses were 560 +/- 20 Å and 950 +/- 20 Å for 2% and 3%, respectively. Adhesion of the lignin film to the substrate was tested by i) 18 hr treatment with 1mM laccase-oxidized ABTS, and ii) submerging the films resulting from i) in 3% H₂O₂ at 60 °C for 1 hr.

Treatment of films with oxidized ABTS. Laccase (25 µg/ml) was added to a 1 mM solution of ABTS in 10 mM sodium lactate buffer pH 4.5 and allowed to catalyze the oxidation of ABTS for 30 min. UV absorbance indicated that under these conditions the maximum concentration of oxidized ABTS is achieved after incubation for 20 min (Fig S9). Following incubation for 30 min, the solution was filtered through a 10,000 g/mol molecular weight cutoff (MWCO) filter to remove the laccase. The lignin films were then incubated against the solution of oxidized ABTS. Following the desired incubation times, the samples were removed and then rinsed thoroughly with Millipore water.

For some assays, the ABTS-treated lignin-coated wafers were cut into samples roughly 5 mm x 5 mm by scribing the back of the wafer.

Film thickness measurements. Film thickness measurements involving individual lignin–coated wafer samples were performed using a Nanofilm EP3 ellipsometer (Accurion GmbH). A minimum of three measurements (at 700° and 532 nm) per sample were collected and averaged. For the multiplexed format, film
thickness measurements were automated using a NanoSpec 6100 spectral reflectometer from Nanometrics, with a wavelength range of 480 – 800 nm. By means of a motorized sample stage and a pre-defined test pattern, the tool semi-autonomously measured each well location (i.e., once the location of the first test site was verified, no further intervention was required). Pre-reaction data were collected with one measurement per well location. Post-reaction data were collected in triplicate—once with the measurement site targeted at the center of the well, again with the site offset slightly above the center line, and a third time offset slightly below the centerline.

**Gel permeation chromatography (GPC).** GPC was performed with as-received lignin and also with lignin recovered from films, either untreated or treated with oxidized ABTS. An Agilent 1200 series binary LC system (G1312B) equipped with DA (G1315D) detector was used. Separation was achieved with a Shodex SB-803 column (6 µm particle size, 300 mm x 8.0 mm i.d., Showa Denko America, Inc., New York, NY, USA) at 80 °C using a mixture of N-methyl-2-pyrrolidinone and dimethyl sulfoxide (1:1 (v/v)) as the mobile phase at a flow rate of 0.5 ml/min. Absorbance of materials eluting from the column was detected at 290 nm. Intensities were area normalized.

**LC-MS.** Aromatic alcohols were analyzed by HPLC — atmospheric pressure chemical ionization (APCI) — time-of-flight mass spectrometry (TOF MS). Separation of these compounds was conducted on an Agilent 1200 Series Rapid Resolution HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) using an Eclipse Plus Phenyl-hexyl column (250-mm length, 4.6-mm inside diameter, and 5-µm particle size; Agilent Technologies, Santa Clara, CA). The mobile phase was composed of 0.1% formic acid in water (solvent A) and methanol (solvent B). The elution gradient used was as follows: from 30% B to 98% B in 20 min, held at 98% B for 5 min, reduced from 98% B to 30% B in 0.5 min, and held at 30% B for a further 3 min. A flow rate of 0.5 mL/min was used until 25 min, after which it was increase to 1 mL/min in 0.5 min and held at this flow rate for an additional 3 min. The total run time was 28.5 min. The column compartment and sample tray were set to 50 °C and 4 °C, respectively. The HPLC system was coupled to an Agilent Technologies 6210 LC/TOF mass spectrometer with a 1:5 post-column split. Mass spectrometric detection was conducted using APCI in the positive ion mode. MS experiments were carried out in the full scan mode, at 0.86 spectra/second, for the detection of [M–H2O+H]+ or [M+H]+ ions. Drying and nebulizing gases were set to 11 L/min and 30 lb/in², respectively, and a drying gas temperature of 330 °C was used throughout. The vaporizer and corona were set to 350 °C and 4 µA respectively, and a capillary voltage of 3,500 V was also used. Fragmentor and OCT 1 RF voltages were each set to 170 V, while the skimmer voltage was set to 50 V. Data acquisition and processing were performed by the MassHunter software package (Agilent Technologies).

Aromatic acids and aldehydes were analyzed by HPLC and electrospray ionization (ESI) TOF MS using a method modified from that described previously,[86] with further detail as follows. Separation of these compounds was conducted via the aforementioned column and HPLC system. A sample injection volume of 5 µl was used throughout. The sample tray and column compartment were set to 4 and 50 °C, respectively. The mobile phase was composed of 10 mM ammonium acetate (Sigma-Aldrich) in water (solvent A) and 10 mM ammonium acetate in 90% acetonitrile and 10% water (solvent B). The mobile phases were made up from a stock solution of 100 mM ammonium acetate and 0.7% formic acid (Sigma-Aldrich) in water. The elution gradient used was as follows: from 30%B to 98%B in 20 min, held at 98%B for 5 min, reduced from 98%B to 30%B in 0.5 min, and held at 30%B for a further 3 min. A flow rate of 0.5 mL/min was used until 25 min, after which it was increase to 1 mL/min in 0.5 min and held at this flow rate for an additional 3 min. The total run time was 28.5 min. The HPLC system was coupled to an Agilent Technologies 6210 series time-of-flight mass spectrometer (for LC-TOF MS) via a MassHunter workstation (Agilent Technologies, CA). Drying and nebulizing gases were set to 11 L/min and 30 lb/in², respectively, and a drying-gas temperature of 330 °C was used throughout. ESI was conducted in the negative ion mode and a capillary voltage of −3,500 V was utilized. MS experiments were carried out in the full scan mode, at 0.86 spectra/second, for the detection of [M–H]− ions. Data acquisition and processing were performed by the MassHunter software package (Agilent Technologies).
Organic acids were analyzed as previously described by Juminaga et al.[87]

UV. UV absorbance spectra were collected on reaction liquids using a JASCO J815 spectrometer. Samples were diluted in Millipore water as necessary.

FTIR. FTIR spectra were collected from lignin-coated wafers using a Bruker Hyperion IR Microscope equipped with a single-bounce germanium attenuated total reflectance (ATR) objective. A total of 512 scans were co-added. Spectral resolution was 4 cm$^{-1}$. Spectra were referenced to air and an atmospheric correction applied to the data to remove water and CO$_2$ vapor contributions. An average of 5 spectra collected from each sample location was used for further analysis and interpretation. Spectra were baseline corrected by fitting a second order polynomial to the spectral region 800-1800 cm$^{-1}$.

FTIR spectra were also collected from residual solid material after evaporating reaction liquids onto Teflon substrates. The material was scraped from the Teflon substrate and pressed against a Pike GladiATR diamond ATR single reflection crystal. IR spectra were collected with a Bruker Equinox 55 IR spectrometer with a DTGS (ID301/8) detector operating at room temperature. A total of 32 scans were co-added. Spectral resolution was 4 cm$^{-1}$. Spectra were referenced to air and an atmospheric correction applied to the data to remove water and CO$_2$ vapor contributions. Spectra were baseline corrected by fitting a second order polynomial to the spectral region.

XPS. XPS was performed using a Kratos Axis Ultra DLD instrument with a monochromatic Al Kα (1486.7 eV) X-ray source operated at 150 W. The analysis area was an elliptical spot of 300 x 700 microns and samples were analyzed at 90° take-off-angle with respect to the analyzer. Base pressures for the instrument were less than 5 · 10$^{-9}$ Torr. Survey spectra were taken with an 80 eV pass energy, 500 meV step, and 100 ms dwell times. High resolution spectra were recorded with a 20 eV pass energy, 100 ms dwell time, and step sizes ranging from 50-100 meV. Data processing was performed with CasaXPS Version 2.3.15.

Cyclic voltammetry. Cyclic voltammetric scans were run using a VoltaLab PGZ402 (Radiometer analytical) in 0.5 M H$_2$SO$_4$ at 100 mV/sec versus an Ag/AgCl and Pt reference and counter electrode respectively.

Water contact angles. Advancing water contact angle measurements were made with a Kruss DSA10-MK2 drop shape analyzer using Millipore water (18.2 MΩ). Reported values are averages of three measurements.

**Multiplexed Format.** A multiplexed format was developed by fabricating a silicone rubber block containing an array of holes in the pattern of a standard 96-well plate. This was achieved by first fabricating a negative mold from a standard 96-well bottomless plastic plate, and then using the mold to fabricate the silicone block with the array of holes. The device (shown in Fig S3) is comprised of an aluminum base plate, a standard 3-in silicon wafer, the silicone block with the array of holes in the pattern of a 96-well plate, the plastic bottomless 96-well plate used to create the mold, and then finally an aluminum top plate with a window cut out to allow access to the wells. Including the plastic bottomless 96-well plate between the silicon part and the aluminum top plate helped to apply a uniform pressure on the silicon form around each hole and prevent leakage between the wells.

For measurements in the multiplexed format, initial thickness measurements were made for a 3-in wafer with lignin film at the locations corresponding to the reaction wells. The device was then assembled, tested for leaks with Millipore water, and then the solutions of FeCl$_3$ and H$_2$O$_2$ were pipetted into each well (300 µl per well). Millipore water adjusted to pH 3 with HCl was used to prepare the solutions. The reactions were initiated by addition of H$_2$O$_2$. An adhesive film was then placed over the top of the wells to minimize evaporation during the reaction. After the desired incubation period, the liquid was removed from each well and frozen until analysis by LC-MS, FTIR, or UV. Immediately after removing the liquid from each well, the well was rinsed three times with 300 µl aliquots of Millipore water to avoid further reaction. The device was then disassembled and the entire wafer rinsed with Millipore water and dried with a stream of nitrogen. For time course measurements, after the liquid from a well was removed at the intended time the well was rinsed two times with 300 µl aliquots of Millipore water and then another 300 µl aliquot of pure Millipore water was left in the well until the completion of the trial. Final thickness measurements were then made using the same grid pattern. Three measurements were averaged for
each spot.

Acknowledgments

This work conducted by the Joint BioEnergy Institute (http://www.jbei.org) was supported by the Office of Science, Office of Biological and Environmental Research, of the U.S. Department of Energy under Contract No. DE-AC02-05CH1123. This work was also supported by the Laboratory Directed Research and Development program at Sandia National Laboratories. Sandia National Laboratories is a multi-program laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the U.S. Department of Energy’s National Nuclear Security Administration under contract DE-AC04-94AL85000. Part of this work was performed at the Center for Integrated Nanotechnologies, a US Department of Energy, Office of Basic Energy Sciences user facility at Los Alamos National Laboratory (contract DE-AC52-06NA25396) and Sandia National Laboratories.

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900 Å film of OS lignin

mass loss quantified by ellipsometry (automated)

300 µl per well

78 simultaneous reactions

mass loss demonstrated with Fenton reaction

Assay effects of: catalytic chemistries, enzymes, organisms