Review

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Characterization and analysis of the molecular weight of lignin for biorefining studies

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Abstract: The molecular weight of lignin is a fundamental property that influences the recalcitrance of biomass and the valorization of lignin. The determination of the molecular weight of lignin in native biomass is dependent on the bioresources used and the isolation and purification procedures employed. The three most commonly employed isolation methods are milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and enzymatic mild acidolysis lignin (EMAL). Common characterization techniques for determining the molecular weight of lignin will be addressed, with an emphasis on gel permeation chromatography (GPC). This review also examines the mechanisms behind several biological, physical, and chemical pre-treatments and their impact on the molecular weight of lignin. The number average molecular weight (M_n), weight average molecular weight (M_w) and polydispersity index (D) all vary in magnitude depending on the biomass source, pre-treatment conditions, and isolation method. Additionally, there is a growing body of literature that supports changes in the molecular weight of lignin in response to genetic modifications in the lignin biosynthetic pathways. This review summarizes different procedures for obtaining the molecular weight of lignin that have been used in recent years and highlight future opportunities for applications of lignin. © 2014 Society of Chemical Industry and John Wiley & Sons, Ltd

Keywords: lignin; average molecular weight; isolation; pre-treatment; characterization

Introduction

The global need for developing renewable, sustainable, transportation fuels continues to grow as demand increases for liquid petroleum products.[1–3] By 2025, the demand on finite petroleum resources is anticipated to increase by 50%.[2] Furthermore, the concern over climate change and net CO_2 increases (Alexander *et al.* (http://www.climatechange2013.org)) has accelerated the need for carbon neutral, sustainable fuels. Thus, extensive research has occurred in the development of biofuels, a renewable and sustainable energy resource.[4,5] The use of lignocellulosic resources such as ag/forest-residues, energy crops and municipal waste streams as the feedstock for developing biofuels is appealing due to its abundance, relatively low cost, and that it does not compete with food.[5,6]

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Cellulose, hemicellulose, and lignin are the three biopolymers that primarily form the plant cell wall. Prior to the fermentation of simple sugars to alcohols, plant polysaccharides need to be enzymatically deconstructed, which requires a pre-treatment to biologically, chemically, and/or physically reduce the recalcitrance of the biomass.[7,8] This recalcitrance is due to the complex structure of the biomass that has evolved to resist the degradation from chemical and biological sources.[4] Pre-treatment technologies involve a complex rearrangement and/or removal of select components of biomass to increase the accessibility and reactivity of cellulose to cellulase.[8] However, it is now well established that cellulase non-specifically absorbs onto lignin which reduces the ability of cellulase to hydrolyze cellulose.[5,9,10]

Lignin is one of the most recalcitrant biopolymers found in the cell wall.[8] It provides strength and rigidity to the plant, helps bind adjacent cells together, and is a key structural component in water transportation due to its hydrophobic properties.[11–13] It also forms lignin-carbohydrate complexes (LCC) in the cell wall making it more difficult to remove, thereby increasing the recalcitrance of the biomass.[7]

The physical structures of the biomass that could inhibit enzyme hydrolysis include the distribution of lignin within the biomass, its structure and its degree of polymerization (i.e. molecular weight).[14] In general, pre-treatment alters the location, content, structure, and/or molecular weight of lignin in the plant cell wall and this may contribute to the reduction of recalcitrance. The significance of the molecular weight of lignin and its relationship to recalcitrance was highlighted in a publication by Ziebell *et al.*[15] As reported in this study, the reduced recalcitrance for a series of transgenic low-lignin alfalfa (*Medicago sativa*) was due, in part, to the lower molecular weight of lignin because of the down-regulation of C3H and HCT genes in the lignin biosynthetic pathway.[15] There appears to be a reduction in reactive monomer species and the non-methoxylated monomers actually terminate chain growth of lignin.[15] It has been reported that an increase in H lignin corresponds with a decrease in chain length and molecular weight; H monomers show an inclination to cap lignin.[15] Smaller lignin could potentially be easier to remove due to being less cross-linked and form fewer bonds with other lignocellulosic polymers in the cell wall.[15] By reducing the molecular weight of lignin, it is possible to decrease the biomass recalcitrance.

In summary, the molecular weight of lignin before and after pre-treatment has become an issue of significant research interest both for understanding the fundamental factors contributing to recalcitrance and for material applications of lignin.[16] Thus, the purpose of this paper is to critically analyze current and future methodological developments used for determining the molecular weight of lignin and to summarize the literature regarding the molecular weight of lignin from various sources at different points in the biorefining process.

Lignin

Lignin is one of the most abundant biopolymers in the world. It usually contributes between 15 wt% and 40 wt% of woody plants' dry matter,[19] as summarized in Table 1. This polyphenolic polymer is primarily derived from

Table 1. Composition of cellulose, hemicellulose and lignin in various biomasses.[17,18]

Biomass	Component (% dry weight)		
	Cellulose	Hemicellulose	Lignin
Switchgrass	34	27	17
Monterery pine	42	21	26
Eucalyptus saligna	48	13	27
Corn stover	37	24	18
Miscanthus[a]	37	36	25

[a]Klason lignin

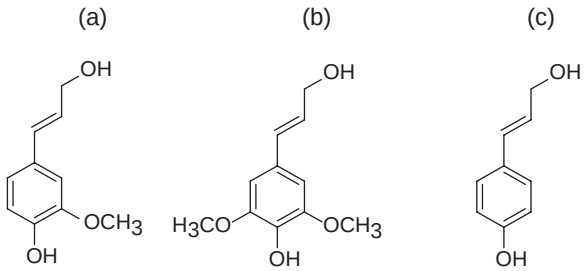

Figure 1. Three monolignol monomers: (a) coniferyl alcohol, (b) sinapyl alcohol, and (c) *p*-coumaryl alcohol.

Table 2. Relative distributions of lignin monomers (%).[15,18,22]

	S	G	H
Poplar (*Populus euramericana*)[a]	63	37	–
Birch (*Betual verrucosa*)[a]	78	22	–
Spruce (*Picea abies*)[a]	Trace	98	2
Miscanthus[b]	44	52	4
Wheat (*Triticum aestivum*)[a]	56	49	5
Alfalfa (*Medicago sativa*)[c]	39	56	5

[a]Thioacidolysis of extractive-free cell walls; [b]MWL; [c]Thioacidolysis and acetyl bromide treatment.

three, or less, monolignols including coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol (Fig. 1).[8,20,21]

These alcohols give rise to the structural units of lignin: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H).[8,19] G lignin is the primary component of softwoods, while hardwoods are composed chiefly of G and S lignin structural units.[8,16,17] H lignin was mentioned in the previous section in relation to alfalfa and the molecular weight of lignin.[15] Table 2 provides some examples the G:S:H values for lignin found in various bioresources. These structural units are connected by various carbon-carbon bonds and ether linkages, the most common being the aryl ether bond, β-O-4.[1,8]

Figure 2 provides a schematic illustration of the inter-unit linkages for softwood lignin, and Sakakibara also provides a comparable illustration.[23] It is important to note that the actual structure of lignin remains unknown.[21]

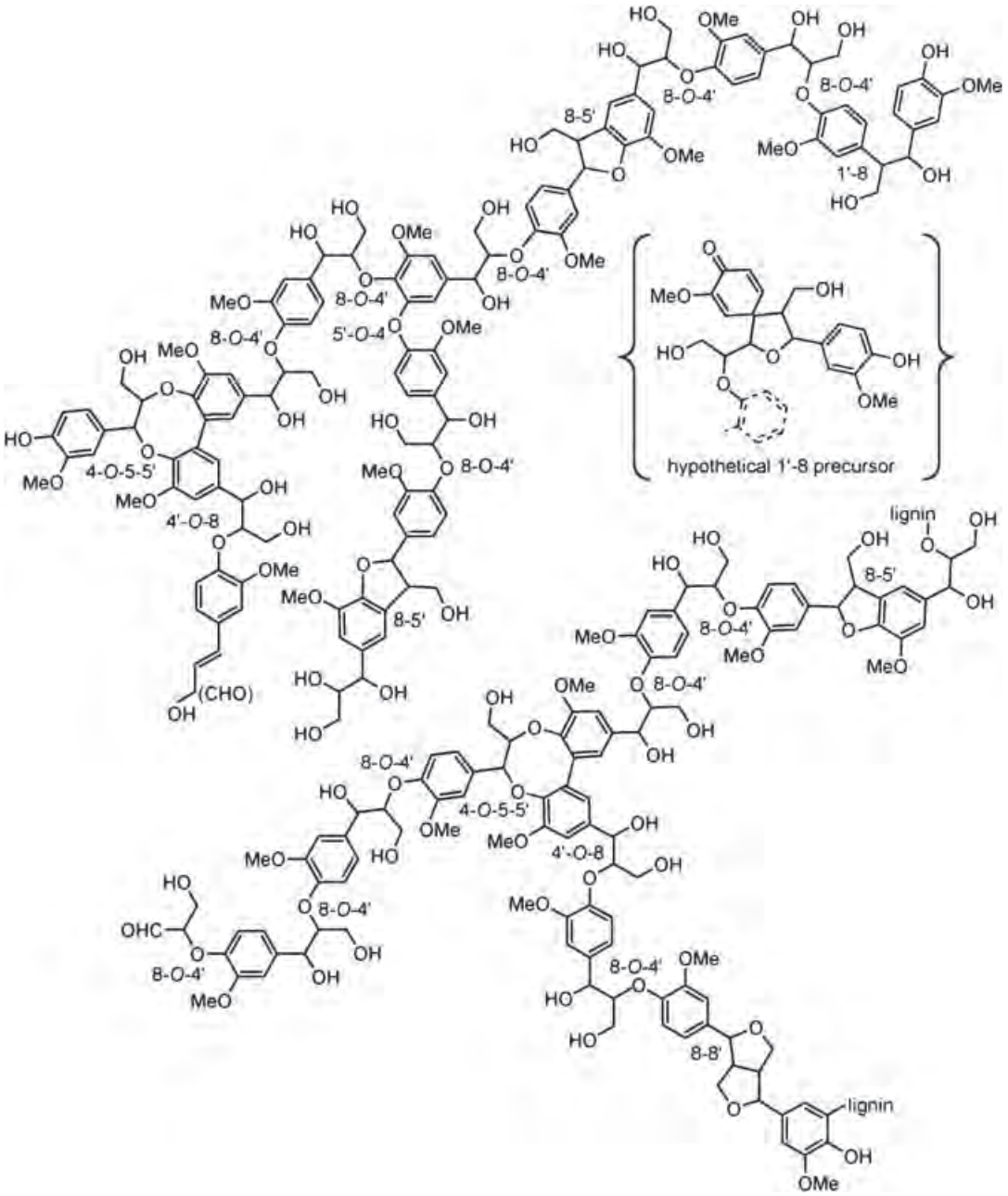

Figure 2. Representation of softwood lignin (public.ornl.gov/site/gallery/detail.cfm?id=131&topic=&citation=&general=lignin&restsection=all).

Definition of average molecular weights of lignin

The non-uniformity of the chain lengths of lignin precludes the characterization of a specific molecular weight. Thus, it is necessary to characterize lignin in terms of average molecular weight. Two common averages used are number average molecular weight (M_n) and weight average molecular weight (M_w) (Eqns (1) and (2)).[24,25] The polydispersity index (D) represents the molecular weight distribution of the polymers (Eqn (3)).[26]

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \quad (1)$$

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \quad (2)$$

$$D = \frac{M_w}{M_n} \quad (3)$$

Here the index number, *i*, represents the number of different molecular weights present in the lignin sample and N_i is the total number of moles with the molar mass of M_i.[24,25]

Isolation of lignin

The isolation of lignin from native biomass is important in allowing for its characterization, including the determination of its average molecular weight. Separating native lignin from cellulose and hemicelluloses needs to be accomplished with minimal structural changes. Milled wood lignin (MWL), cellulolytic enzyme lignin (CEL), and enzymatic mild acidolysis lignin (EMAL) isolation methods (Table 3) will be addressed in greater detail along with corresponding average molecular weights.

Milled wood lignin

Most non-enzymatic lignin isolation techniques used today minimize changes in the structure of lignin and use a ball-milled sample to isolate MWL.[27,28] Milling wood/biomass helps maximize extracted lignin; milling can be accomplished by using a vibratory ball-mill or rotary ball-mill to reduce biomass particle size to 0.8 mm or less.[28–30] Björkman determined that the vibratory ball-milling was improved when toluene was added to the process to prevent the milled wood from adhering to the steel balls.[29] Essentially, he developed a process that would isolate lignin at room temperature by extracting ball-milled wood using neutral solvents (Table 3).[29] Björkman compared the use of aqueous *p*-dioxane and an ethylene chloride/ethyl alcohol mixture as the solvents for the extraction process showing that the former solvent provided approximately a six-fold increase in yield.[31] Both toluene and N_2 atmosphere were determined by Holtman *et al.* to be preferred when preparing vibratory ball-milled MWL as they could minimize side reactions and condensation reactions.[28] In addition, they used porcelain jar and balls for the rotary ball-milling and extended the time of milling to six weeks which resulted in optimal particle size (~0.84 mm) for lignin extraction.[28] Guerra *et al.* also used a porcelain jar and ball for the rotary ball-milling, and showed that obtaining high molecular weight lignin fragments increased for longer ball-milling time.[32] Other reports indicate that an increase of milling time from four weeks to six weeks increased the yield by approximately 8-10% for MWL.[33] The ball-milling process is known to be accompanied with mechanochemical changes in lignin such as the cleavage of some β-O-4 linkages.[34] Fujimoto *et al.* report that once approximately 40% of the lignin has been

Table 3. Overview of experimental procedures used for lignin isolation.

Milled Wood Lignin[29,31]	1. Ball-milled wood is extracted with aq. p-dioxane (4% water) at RT 2. Extracts are dried and dissolved in acetic acid 3. Precipitated into water 4. Dried, then dissolved in ethylene chloride and ethanol, followed by precipitation into diethyl ether
Cellulolytic Enzyme Lignin[34]	1. Ball milled biomass added to cellulase, incubate for 3 days 2. Wash with water and extract twice with aq. p-dioxane 3. Dissolve in acetic acid and precipitate into water 4. Isolate lignin and wash twice with water, suspend in water and freeze-dry
Enzymatic Mild Acidolysis Lignin[32]	1. Treat ball-milled wood with cellulase 2. Shake in a water bath using citrate buffer (pH 4.5) 3. Wash soluble material with acidified DI water twice and freeze-dry 4. Treat cellulytic lignin with aq. p-dioxane 5. Filter and neutralize with sodium bicarbonate and add to acidified DI water, leave over night 6. Remove precipitated lignin, wash with DI water twice and freeze-dry

Table 4. Typical lignin yields from various biomass sources isolated by MWL, CEL, and EMAL.[27,32]

Biomass	Approximate Isolation Method Yields[a] (%)		
	MWL	CEL	EMAL
Norway Spruce (milled)	11.4	23.4	44.5
Douglas Fir	1.4	7.1	24.8
Redwood	15.7	13.2	56.7
White Fir	11.3	11.5	42.9
E. globulus	34.0	32.5	63.7
Southern Pine	11.9	12.4	56.3

[a]Basis of extracted ground wood meal Klason lignin contents

extracted via milling, there is about a 25% reduction of β-O-4 bonds.[35]

Milling allows for more lignin extraction due to the disruption of the structure of the cell wall.[32] Lignin is initially released from the middle lamella and cell corners during milling primarily because these regions contain high lignin content.[30] The secondary wall of the cell typically has a lower degree of condensation compared to these regions.[30] The degradation of carbohydrates helps in the isolation of CEL which indicates that the lignin primarily originates from the secondary cell wall;[30] MWL contains more condensed lignin from the middle lamella which can result in low yields for MWL.[27] The lower yields of MWL can be compared with the other isolation methods in Table 4. When looking at the lignin yields, it is clear that the yields from EMAL isolation are at least two times that of MWL or CEL and almost four times greater as in the case of white fir. These results show that despite the varying biomass sources, EMAL isolation continues to produce the highest lignin yields. It is important to note that despite any structural changes to the lignin during the MWL isolation process, MWL is generally viewed as representative of native lignin's chemical structure and reactivity.[35]

Cellulolytic enzyme lignin

An alternative lignin isolation method, developed by Chang *et al.*, involves removing a substantial amount of the carbohydrate fraction with a cellulolytic enzyme treatment before aqueous *p*-dioxane extraction of mildly ball-milled biomass yielding CEL[34] (Table 3). This method was devised to improve the lignin isolation process using enzymes with cellulolytic and hemicellulolytic activities, such as Onozuka SS1500 Cellulase.[34,36] CEL typically results in lignin yields higher than MWL, but there are a few bioresources, like redwood, that have a lower yield for CEL (Table 4). While CEL may contain some protein impurities and carbohydrates, it does provide a good resource for lignin structural analysis.[35] The presence of the carbohydrates most likely comes from the lignin-carbohydrate complexes due to covalent bonds between lignin and hemicellulose.[37] Table 5 documents common carbohydrates found in biomass that could contribute to the fraction found with the isolated lignin.

Along with the carbohydrates, there are some protein contaminants associated with CEL that have been attributed to the enzymes used in hydrolyzing the cellulose.[41] Ibara *et al.* used a protease treatment to purify the residual lignin they obtained from eucalyptus unbleached kraft pulp.[41] Protease hydrolysis (2% alkaline protease) purified the lignin of the cellulase residue and resulted in a recovery of 98% of the total lignin, but it still contained some protein contaminants.[41] They also reported a solvent purification method, using dimethylacetamide and sodium hydroxide, that purifies the residual lignin, but only obtained a 1–3% total yield.[41] Lou *et al.* showed that a reduction of non-specific cellulase binding to lignin was a result of inducing pH surface modifications to the lignin.[42] When the pH was greater than or equal to 5.5, the enzymatic saccharification of lignocellulose was enhanced.[42] Thus, the protease technique is the desired process to minimize the cellulase residue due to the high resulting yield.

Table 5. Carbohydrate relative composition in various biomasses (% dry weight).

	Hybrid Poplar[17]	*Miscanthus*[a,38]	Wheat Straw[39]	Rice Straw[39]	Switchgrass[b,40]
Arabinan | Arabinose	0.89 |	1.83 |	| 5.6	| 5.4	3.9 |
Galactan | Galactose	0.88 |	0.43 |	| 2.7	| 7.1	1.3 |
Glucan | Glucose	39.23 |	49.09 |	| 55.4	| 57.7	33.6 |
Mannan | Mannose	1.81 |	0.27 |	| 1.5	| 2.1	0.1 |
Xylan | Xylose	13.07 |	21.65 |	| 34.6	| 27.6	24.7 |

[a]Without prehydrolysis; [b]Extracted with hot H_2O and C_6H_6/EtOH.

Enzymatic mild acidolysis lignin

Another procedure for isolating lignin involves a mild enzymatic hydrolysis preceding a mild acid hydrolysis of milled wood, resulting in EMAL.[27,32] The enzymatic hydrolysis of milled wood removes a significant amount of carbohydrates, while the mild acidolysis cleaves lignin-carbohydrate bonds.[32] The acid hydrolysis step is important because lignin-carbohydrate complexes impede lignin isolation in high yields.[27,43,44] Combining this procedure (Table 3) with low severity milling, results in high yields of less modified lignin.[43,44] The EMAL procedure yields are approximately 2 to 5 times greater than the MWL or the CEL procedure yields, as summarized in Table 4.[27,32] Guerra *et al.* even reported that EMAL isolated lignin fractions that the other lignin isolation procedures were unable to obtain.[32]

Average molecular weight after isolation processes

The number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (D) of MWL, CEL, and EMAL for various biomass sources can be found in Tables 6–8. Select biomasses with the largest M_n are *miscanthus* for MWL (8300 g mol^{-1}), Norway spruce for CEL (9450 g mol^{-1}), and Southern pine for EMAL (9700 g mol^{-1}). Norway spruce reported the largest M_w for MWL, CEL, and EMAL at 23,500 g mol^{-1}, 53,850 g mol^{-1}, and 78,400 g mol^{-1}, respectively.

Looking at Tables 6–8, the general trend for the M_w for lignin isolation is EMAL > CEL > MWL. The data indicates that EMAL isolation results in higher M_w for the various biomasses. The higher molecular weight for EMAL over MWL and CEL can be attributed to its isolation process. Guerra *et al.* determined a higher M_w for EMAL correlates with a lower vibratory milling time.[32] By adding 94 h of additional milling time to the original 2 h, the EMAL M_w decreases by approximately 40,000 g mol^{-1} and is only 2650 g mol^{-1} greater than CEL M_w.[32] Overall, EMAL appears to produce the highest M_w compared to MWL and CEL.

It is interesting to note that M_n has a similar trend to M_w for the isolation processes. The exceptions are Norway spruce and redwood, where the M_n for CEL is greater than that of EMAL by 600 g mol^{-1} and 700 g mol^{-1}, respectively. While M_n is not as clear as M_w for which isolation process will typically result in the highest molecular weight, EMAL does consistently report relatively high M_n values.

Note that we will be referring to the average molecular weight as molecular weight throughout the rest of the paper.

Table 6. Average molecular weights and polydispersity indices from milled wood lignin of various biomasses.[18,27,32,45]

Biomass	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Norway Spruce[a]	6400	23,500	3.7
Douglas Fir[b]	2500	7400	3.0
Redwood[b]	2400	5900	2.5
White Fir[b]	2800	8300	3.0
E. globulus[b]	2600	6700	2.6
Southern Pine[b]	4700	14,900	3.2
Bamboo	5410	12,090	2.23
Miscanthus[c]	8300	13,700	1.65

[a]Vibratory-milled; [b]Ball mill for 28 days; [c]Values corrected from the original manuscript after a personal discussion with the author.

Table 7. Average molecular weights and polydispersity indices from cellulolytic enzyme lignin of various biomasses.[27,32]

Biomass	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Norway Spruce[a]	9450	53,850	5.7
Douglas Fir[b]	5500	21,800	4.0
Redwood[b]	5400	23,000	4.2
White Fir[b]	4700	21,700	4.6
E. globulus[b]	5500	17,200	3.1
Southern Pine[b]	7500	29,600	3.9

[a]Vibratory-milled; [b]Ball mill for 28 days prior

The instrumental analysis of the molecular weight of lignin

The characterization of the molecular weight of lignin

Vapor pressure osmometry (VPO),[46] ultrafiltration,[47] light scattering (static and dynamic),[48] mass spectrometry,[49] and gel permeation chromatography (GPC)[50,51] represent a pool of instruments available to characterize the molecular weight of lignin. Among these methods, GPC has grown in popularity and will be discussed in further detail. Accordingly, Table 9 summarizes the basic principles of the former techniques. GPC, also known as size exclusion chromatography (SEC), separates derivatized or underivatized lignins based on molecular size in order to determine its relative molecular weight.[52] Universal calibration[53] and broad standard calibration[54,55] offer two means of molecular weight determination using GPC.[56]

Table 8. Average molecular weights and polydispersity indices from enzymatic mild acidolysis lignin of various biomasses.[27,32]

Biomass	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Norway Spruce[a]	8850	78,400	8.8
Douglas Fir[b]	7600	38,000	5.0
Redwood[b]	4700	30,100	6.4
White Fir[b]	6300	52,000	8.2
E. globulus[b]	8700	32,000	3.7
Southern Pine[b]	9700	57,600	5.9

[a]Vibratory-Milled for 48h; [b]Ball mill for 28 days prior

The attractiveness of GPC is attributed to short sample processing times (30 min to 5 h per sample), the milligram quantities of sample required for testing, its tolerance of synthetic and naturally occurring polymers, and the broad range of molecular weight detection.[60,61] Glasser *et al.* successfully collected the M_n and M_w of MWL, acid-hydrolyzed, steam-exploded, kraft and organosolv lignins, establishing that GPC is practical for several lignin types.[62] Futhermore, the comparison of GPC to other techniques supports its reliability. The M_w and M_n of polystyrenes that were acquired from matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) have been compared to those obtained from VPO, GPC, light scattering, and intrinsic viscosity to assess the accuracy of MALDI-TOFMS in the determination of molecular weight. While it was recognized that a polymer's molecular weight relies upon several factors, each method provided similar estimations of molecular weight.[63]

While several methods measure the molecular weight of lignin, each method differs in its capacity. The differentiating feature of light scattering as compared to other methods is its overrepresentation of heavier lignins.[64,65] VPO, however, is sensitive to the presence of compounds possessing molecular weights < 25,000 kDa.[66] Froment and Pla investigated the application of VPO, SEC, and LALLS (low angle laser light scattering) in the molecular weight determination of dioxane, alkali, and organosolv lignins. The results emphasized the practicality of VPO as a colligative method for the calculation of M_n, and the appropriateness of LALLS in the calculation of the absolute values of M_w. SEC, which computes both M_n and M_w, when coupled with LALLS introduces an enhanced approach to molecular weight determination.[66] GPC and MALDI-TOFMS generally produce similar molecular weights; GPC, however, slightly overestimates these values.[67] Furthermore, it has been recognized that MALDI-TOFMS is appropriate for the molecular weight determination of low polydispersity polymers (D < 1.2), while GPC is more suitable in the molecular weight determination of polymers of greater polydispersities. GPC is paired with other instruments such as MALDI-TOFMS[68] and static LALLS[69] or MALLS[70] to provide more accurate values of molecular weight.

Gel permeation chromatography as a tool for molecular weight determination

Prior to analysis, derivatization is typically accomplished through methylation, acetylation, or silylation to enhance lignin's solubility in an organic solvent.[52,71] The length of time required to achieve the complete dissolution of acetylated lignin in organic solvents using conventional methods remains in question. Studies have identified acetylation as suitable for the dissolution (> 90%)[51] and complete dissolution[72] of lignin from several biomass sources in THF. However, Assikkala *et al.* found that only 54% of acetylated Norway spruce MWL dissolved in THF after a 20 h treatment, and this rose to 60% after 6 d.[73]

Acetobromination[73] and ionic liquid media[74] offer alternative approaches to conventional derivatization. The

Table 9. Characterization methods for the molecular weight of lignin.

Analytical Technique	Principle	Relevant Information
VPO[52]	Thermistors indirectly assess the vapor pressure depression of a dilute polymer solution compared to a pure solvent to extrapolate M_n	Exhibits sensitivity towards low molecular weight polymers M_n=100–10,000 g mol^{-1}
Ultrafiltration[52]	Membrane separates lignin based on cutoffs that restrict passage by molecular size	Not influenced by impurities M_n=1000–300,000 g mol^{-1}
Light Scattering[52,57]	Laser beam passes through a sample and scatters light at an intensity that is proportional to its molecular weight	Static LS provides an absolute determination of molecular weight, whereas dynamic LS requires calibration
Mass Spectrometry[58,59]	Electron converts a gaseous neutral molecule into a radical cation, which possesses a specific mass and charge	Sensitive to highly polydisperse lignin

acetobromination of Norway spruce MWL with acetyl bromide in glacial acetic acid achieved complete dissolution in 0.5 h.[73] Underivatized lignins dissolve in solvents such as dimethylformamide and dimethylsulfoxide but are susceptible to association effects, which potentially reduce the reproducibility and reliability of molecular weight data.[73,75,76] The mechanisms and presence of association effects have been extensively studied using GPC.[43,54,76] Lithium bromide or lithium chloride is often added to DMF or DMSO to minimize the association effects.[75] Majcherczyk and Hüttermann used high-performance ion-pair size-exclusion chromatography with styrene–divinylbenzene gel columns and a quaternary amine (QAM) to establish the molecular weight of organosolv and kraft lignins in tetrahydrofuran without derivatization. The QAM formed complexes with lignin, which successfully minimized column adsorption and lessened association effects.[77]

The derivatized or underivatized lignins are then dissolved in the appropriate eluent and introduced to a porous gel column, where size separation is accomplished. The hydrodynamic radius of lignin dictates whether a lignin molecule is included or excluded from a pore in the gel. The lignin then elutes from the column at a volume related to its size and the dimensions of the pores in the gel. The elution volume is coupled with a standard to determine relative molecular size and weight.[52] Polystyrene standards are often used to construct the calibration curve for GPC measurements that require organic solvents; their molecular weights range up to 10,000,000 g mol^{-1} with polydispersities below 1.10.[56] The hydrodynamic volumes of lignin and polystyrene differ, indicating that GPC is a relative method.[52]

The estimation of the molecular weight of lignin using GPC is reliable on an aqueous or non-aqueous GPC as well as in the presence of a polystyrene standard or lignin-based standard for the construction of a standard curve. Chen and Li compared the reliability of an aqueous GPC for the molecular weight determination of lignins to a non-aqueous GPC. The molecular weights of Eucalyptus soda lignins were in good agreement between apparatuses; the maximum variation between M_n and/or M_w was roughly 1000 g mol^{-1}. In addition, the M_n of lignosulfonates determined by the aqueous GPC and VPO were also in good agreement with sodium lignosulfonate yielding a difference of less than 40 g mol^{-1}, supporting the hypothesis that the aqueous GPC produces reliable estimations of molecular weight. While the agreement amongst methods was present, pH and ionic strength did influence the reliability of the method.[78] VPO also supports the use of acetosolv lignins with polydispersities of approximately one as calibration standards in place of polystyrene for GPC. The acetosolv lignins selected for the construction of the calibration curve produced a maximum difference in M_n of 500 g mol^{-1} between methods. Moreover, the calibration curves of polystyrene and acetosolv lignins were superimposed on one another almost perfectly.[79] A similar study verified that roughly monodisperse alkali lignins were fitting for the preparation of a GPC calibration standard curve; the M_w of the lignosulfonate calculated from the standard curve was 17,600 g mol^{-1}, which was 700 g mol^{-1} less than the value calculated by ultracentrifugation.[80]

Pre-treatments and the analysis of M_n, M_w, and D of lignin

As previously stated, pre-treatments are divided into three categories: biological, physical, and chemical. Within each category, numerous approaches and pre-treatment conditions have been developed and studied (Table 10). The literature commonly compares extracted MWL, a rough model of native lignin, to lignin isolated from pre-treated biomass to assess changes in molecular weight in terms of M_n, M_w, and D (Table 11). For instance, the comparison of the molecular weight of MWL (~10^4 g mol^{-1}) against lignin extracted from steam exploded (~10^4 g mol^{-1}) and autohydrolyzed (~10^3 g mol^{-1}) aspen (*Populus tremula*) revealed that autohydrolysis yielded a 10-fold reduction in molecular weight at the severity studied.[81] CEL and EMAL serve as references to lignin isolated from pre-treated biomass (Table 12). MWL, CEL, and/or EMAL are often compared to lignin from pre-treated biomass to assess the changes in molecular weight, as demonstrated in Tables 11 and 12. The application of a pre-treatment does not guarantee the molecular weights produced are lower or higher than that of MWL, EMAL, or CEL. For instance, steam explosion generally produces values of M_w that are greater than MWL, while pre-treatments such as ethanol organosolv, dilute acid, and alkali often produce values of M_w that are similar to the values of MWL. Tables 11 and 12 also indicate that pre-treatments exhibit various degrees of modifications to the molecular weight and hence the structure of lignin.

Lignin in the pre-treatment effluent is frequently recovered through acid precipitation. The fraction of lignin that is regenerated through precipitation is acid insoluble, whereas the remainder of lignin in the effluent is acid soluble.[82] The molecular weights reported hereafter describe lignins that have been separated and isolated during or

Table 10. Select pre-treatments, their classifications, and processing conditions.†

Pre-treatment	B, P, or C[a]	Conditions
Organosolv[83,93]	C	180–195˚C, 30–90 min, 35–70% ethanol
Steam Explosion[83]	P, C	Saturated steam at 160–260˚C and 0.69–4.83 MPa initially, followed with a rapid decrease in pressure
Liquid Hot Water[84]	P, C	Liquid water at 160–240°C
Supercritical Water[85,86]	C	< 373˚C and < 22 MPa, approximately
White rot Fungi[87]	B	Biomass containing 60-80% moisture, pH 4–5, 15–35˚C, incubate for d, wks, or mo
Alkaline (NaOH, KOH, $Ca(OH)_2$, NH_4OH)[88]	C	25–85˚C, 1–30 h
Dilute Acid[89]	C	0.3–2 wt% acid, 120–180°C, 1 min to 2h+
Ammonia Fiber Explosion (AFEX)[83,90,94]	P, C	1–2 kg liquid NH_3/kg dry biomass, 60–100˚C, 10–60 min, > 3 MPa
Wet Oxidation[91]	P, C	1L H_2O/6 g biomass, 1.2 MPa, 195°C, 10–20 min
Acid- or SO_2- Catalyzed Steam Explosion[92]	P,C	0–5% OD, 190–210°C for hardwoods, 200–220°C for softwoods, 1–10 min
Sulfite Pre-treatment to Overcome Recalcitrance of Cellulose (SPORL)[92]	C	Sodium, calcium, or magnesium bisulfite, 160–190°C, pH 2–5
Ionic Liquid[93]	C	20–150˚C, 1–48 h
Carbon Dioxide (CO_2) Explosion[94]	P,C	Apply supercritical CO_2 at 6.8–27.6 MPa, several min, < 200˚C

†Herein, the conditions listed in this table serve as an approximate guide for subsequent molecular weights for lignin.
[a]B: biological; P: physical; C: chemical.

Table 11. The M_n, M_w, and D of MWL and lignins isolated from pre-treated biomasses.

Biomass	Pre-treatment	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Cotton Stalk[95]	MWL	700	1520	2.17
	Ammonia Hydrothermal	560–890	1250–1740	1.83–2.23
Bamboo (*Bambusa rigida* sp)[96]	MWL	1680	3260	1.93
	AL	1860	2840	~1.5
Birch (*Betula alnoides*)[97]	MWL	5860	10,860	1.85
	Microwave	3830	7290	1.90
	Heat	5000	11,450	2.29
Beech (*Fagus sylvatica*)[98]	MWL	3690	5510	1.49
	Heat	2790	4020	1.44
Loblolly Pine (*Pinus taeda*)[99, 100]	MWL	989	7790	7.9
	Ceriporiopsis subvermispora	743–770	5147–6330	6.7–8.5
	MWL	7590	13,500	1.77
	OS-MWL	6530	16,800	2.57
	EOL	3070	5410	1.77
Poplar (*Populus trichocarpa*)[101]	MWL	–	8550	2.7
	DAP	–	7500–8280	2.2-3.0
Switchgrass (*Panicum virgatum* var. Kanlow)[40]	MWL	2070	5100	2.5
	EOL	980	4200	4.3
	Ethanol organosolv + ball mill	1580	5750	3.6
Poplar (*Populus albaglandulosa*)[102]	MWL	4176	13,250	3.17
	Supercritical H_2O	1042–1357	1655–4429	1.59–3.26
	Supercritical H_2O + catalyst	949–1097	1526–2753	1.55–2.63
Tamarix ramosissima[103]	MWL	2155	3750	1.74
	LHW	1380–2250	2690–3950	1.76–1.95
Lodgepole Pine Wood Chips[104]	SPORL (LS-SP165)	810	1440	1.77
Commercial Softwood[104]	SPORL (LSD-748)	4800	14,000	2.92

AL: alkali lignin; DAP: dilute acid pre-treatment; EOL: ethanol organosolv lignin; LHW: liquid hot water; OS-MWL: organosolv milled wood lignin; SPORL: sulfite pre-treatment to overcome recalcitrance of lignocellulose.

Table 12. The M_n, M_w, and D of EMAL and CEL in comparison with lignins isolated from spruce, wheat straw, birch, and poplar.

Biomass	Isolation/ Pre-treatment	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Spruce (*Picea abies*)[105]	EMAL	3100	13,700	4.4
	EMAL + steam explosion	3800	21,500	5.6
Wheat Straw[105]	EMAL	2000	3600	1.8
	EMAL + steam explosion	2700	6100	2.2
Birch[106]	CEL	7810	18,300	2.51
	ILL	4820	7780	1.62
	RL	5970	8900	1.49
Poplar (*Populus tomentosa* Carr.)[107]	CEL	980	3190	3.26
	AL	1510	2330	1.54
	AL + [C2mim][OAc]	2520	3970	1.58

AL: alkali lignin; [C2mim][OAc]: 1-ethyl-3-methylimidazolium acetate; ILL: ionic liquid lignin; RL: residual lignin.

after the pre-treatment of biomass; the original reference should be consulted for a detailed description of the recovery procedure.

Biological pre-treatments

White rot fungi, a commonly studied biological pre-treatment, uses lignolytic enzymes to degrade cell wall components including lignin. For example, *Ceriporiopsis subvermispora* possesses laccases and manganese peroxidases, which assist in the selective removal of lignin during the initial phases of pre-treatment. *C. subvermispora* removed 45.06% of lignin and 9.50% of cellulose in rubberwood (*Hevea brasiliensis*) after 90 d of incubation.[108] Furthermore, *C. subvermispora* successfully digested lignin from *Pinus taeda*, which was indicated by the 35% decrease in molecular weight compared to MWL (M_w 7790 g mol^{-1}) following 90 d of incubation.[99] Biomass composition may also regulate enzyme activity. Poplar induced up to 6 times the laccase activity of *C. subvermispora* than pine and wheat straw over 22 d at 30°C.[109] Poplar, a hardwood, contains both S and G monomers; degradation, or the lowering of molecular weight, of S lignins occurs more readily than G lignins.[109] Lignin from wheat straw demonstrated a time-dependent decrease in M_w when treated with *Fusarium concolor*, another type of fungi. The wheat straw lignin at day 30 was 25% less than the M_w at day five.[110]

Bacteria constitute another type of biological pre-treatment that enzymatically remove lignin.[111] During decomposition, fungi initially break down native lignin. Afterwards, bacteria such as *Streptomyces viridosporus* strain T7A[112] mineralize the lower molecular weight decomposition compounds that remain.[113] Crawford *et al*. identified an acid-precipitable polyphenolic polymeric lignin intermediate, possessing a molecular weight greater than or equal to 20,000 g mol^{-1}, present during the breakdown of corn stover (*Zea mays*) using *S. viridosporus*.[114] *Pseudomonas putina* and *Rhodococcus RHA1* are bacteria present in soil that also degrade *Miscanthus*. Following seven days of incubation at 30°C, these bacteria produced ketone and carboxylic acid containing byproducts, which possessed roughly 1/6th the original mass-to-charge ratio of MWL.[115] Fungi and bacteria are suitable methods to modify the molecular weight of lignin; however, the long incubation times required to achieve degradation add reservations to its industrial viability.

Physical pre-treatments

Physical pre-treatments, such as microwave, also remove lignin.[116] Microwave pre-treatments, specifically, expose biomass to microwaves, which stimulate the vibration of polar molecules and the motion of ions. The movement of ions produces heat and enhances collisions that lead to mass transfer processes and the dissolution of biomass. In sweet sorghum bagasse, microwave irradiation alone removed roughly 20 to 30% of lignin following 2 to 6 min of exposure to 1000 W of microwaves. Increasing the volume of water from 10 to 20 mL per g of biomass during pre-treatment shifted the percentage of lignin removed upward towards 30%.[116] Microwave irradiation is often paired with other pre-treatments to enhance its delignifying abilities.[97,117–119] The microwave-assisted ethanol organosolv extraction of lignin from triticale (*Triticosecale X*) straw resulted in a substantially larger decrease in the molecular weight of residual lignin when compared to the extracted lignin from conventional heating in the presence of an acid catalyst; the difference between the two methods was 5501 g mol^{-1}.[118]

Chemical pre-treatments

Chemical pre-treatments encompass a broad spectrum of chemicals that include oxidizing agents, acids, alkalis, solvents, and sulfite as in SPORL.[120,121] Organosolv pre-treatments, for example, fragment and solubilize lignin using an organic solvent system, especially when coupled to a catalyst such as a mineral acid or sulfate salt. Organosolv pre-treatment assists in cleavage of bonds between lignin and hemicelluloses as well as select inter-unit linkages between lignin.[93] Increases in ethanol concentration

from 65 to 95% are associated with carbohydrate content decreases that range from 3.6 to 1.1%, respectively.[122] In addition to ethanol concentration, the changes to the combined severity factor alter the molecular weights of lignin. During the organosolv pre-treatment of the wetland plant *Typha capensis*, an increase in severity factor from 1.36 to 2.14 resulted in a decrease of 245 g mol^{-1} in M_w, demonstrating a relationship between treatment severity and the resulting molecular weight.[123]

Studies have also examined process parameters that may alter molecular weight of lignin during the organosolv pre-treatment. Table 13 outlines two studies that examine process parameters during the organosolv pre-treatment that influence the molecular weights of lignin. The table indicates that the choice of organic solvent (i.e. ethanol, acetone, and p-dioxane) minimally influences the molecular weight of the residual lignins recovered from *Miscanthus giganteus* under 4 h reflux.[122] Organosolv pre-treatments can be combined with other chemicals or pre-treatments[124] to exert a more drastic effect on molecular weight.[40] For example, the treatment of *Miscanthus* with autohydrolysis or the enzyme Cellulyve® prior to an ethanol organosolv pre-treatment lowered the M_w and M_n up to 25 and 32%, respectively, when compared to organosolv lignin alone.[38] Furthermore, ultrafiltration separated the molecular weights of ethanol organosolv lignins with ceramic membranes possessing 5, 10, and 15 KDa cutoffs and refined the polydispersities of organosolv lignins in *Miscanthus sinesis*.[125] Table 14 presents the molecular weights of lignin from biomass and their relation to different pre-treatments. This data demonstrates occasions when biomass sources treated with the same processing conditions yield drastically different molecular weights and other instances when different processing conditions or types yield similar molecular weights.

Acidic and basic pre-treatments

Dilute acid pre-treatment (DAP) employs mineral acids at concentrations of 0.3–2 wt% typically between 120 to 180˚C for 1 min to over 2 h to remove hemicelluloses and disrupt the structure of lignin.[89,129] DAP primarily hydrolyzes hemicelluloses and partially solubilizes lignin. Cao *et al.* demonstrated that DAP produces minimal changes in the molecular weight of lignin in *Populus trichocarpa* sampled between 0.3 and 26.8 min.[101] Under certain conditions such as in batch processes, DAP hydrolyzes β-O-4 linkages in lignin.[130] However, these hydrolysis reactions are accompanied with the formation of condensed phenolics.[130] Degradation products from carbohydrates and lignin generated during DAP impede enzyme activity in certain instances; however, the regulation of pH, pressure, and heat restricts the production of these undesirable byproducts.[129] During alkali pre-treatment, a caustic reagent cleaves benzyl ester and glycosidic bonds in LCCs. Alkali reagents include sodium hydroxide, potassium hydroxide, ammonia, and calcium hydroxide (lime). Alkali pre-treatments change the structure of lignin, induce the swelling of cellulose, and solvate hemicelluloses which can promote enzyme accessibility.[131]

The combination of alkali and organosolv pre-treatments incurred more drastic changes in molecular weight than using alkali alone. The residual lignin extracted from bamboo using 70 wt% ethanol that contained NaOH was roughly 1000 g mol^{-1} higher in M_w than those extracted using NaOH alone. The difference in molecular weight was attributed to the presence of intact LCCs in the organosolv

Table 13. The M_n, M_w, and D of isolated lignins as a function of process variables.

Biomass	Pre-treatment	Conditions	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Miscanthus[38]	Organosolv	80% EtOH, 0.5 wt% H_2SO_4, 150°C, 60 min	1932	5715	2.96
	Autohydrolysis + organosolv	Autohydrolysis at, 150°C, 8 h then organosolv	1496	5103	3.41
	Enzymatic hydrolysis + organosolv	Enzyme hydrolysis at, 5% Cellulyve®, 50°C, 72 h then organosolv	1316	4281	3.25
Miscanthus giganteus[122]	Ethanol organosolv	65% ethanol/5% 0.2 M HCl, 4 h, reflux	1520	2720	1.79
		75 % ethanol/5% 0.2 M HCl, 4 h, reflux	1450	2540	1.75
		85% ethanol/5% 0.2 M HCl, 4 h, reflux	1400	2460	1.76
		95% ethanol/5% 0.2 M HCl, 2 h, reflux	1350	2210	1.64
		95% ethanol/5% 0.2 M HCl, 4 h, reflux	1240	2250	1.81
		95% ethanol/5% 0.2 M HCl, 8 h, reflux	1170	2380	2.03
	Acetone organosolv	95% acetone/5% 0.2 M HCl, 4 h, reflux	1420	2170	1.53
	Dioxane organosolv	95% dioxane/5% 0.2 M HCl, under N_2, 4 h, reflux	1240	2310	1.86

EtOH: ethanol; H_2SO_4: sulfuric acid; HCl: hydrochloric acid; NaOH: sodium hydroxide.

Table 14. The variation of M_n, M_w, and D of lignin with biomass and pre-treatment type.

Biomass	Pre-treatment	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Siam weed[126]	Acetosolv	710	2010	2.83
	Milox	720	2100	2.92
Triticale Straw[127]	Microwave-assisted ethanol organosolv	798	2320	2.9
Wheat Straw[127]		780	2015	2.6
Corn Residue[127]		842	2609	3.1
Flax Shives[127]		1408	4500	3.2
Hemp Hurds[127]		1054	2418	2.3
Rye Straw[128]	Organosolv	–	8680	5.1
	Soda pulping	–	8000	4.8
	Aquasolve	–	2600	3.1
	Aquasolve + enzymatic hydrolysis	–	7080	4.0

lignin (4.5% total sugars) as compared to the alkali lignin (0.22–0.94% total sugars); the carbohydrates attached to lignin increase the hydrodynamic volume, and hence the apparent molecular weight of residual lignin.[132] The molecular weight of lignin also increases following alkali pre-treatment when coupled with ultrasonication. The application of 20 min of ultrasonication to alkali lignin extracted from wheat straw (*Variety riband*) increased the molecular weight by 20% as compared to 5 min, which was attributed to condensation reactions.[133]

Green solvents

Green solvents such as ionic liquids (ILs) and supercritical fluids are alternative options to pre-treat biomass. ILs represent salts that are liquids at temperatures below 100°C.[91] Supercritical (SC) solvents, such as SC-carbon dioxide and SC-water, are fluids that exist in a state above their critical temperature and pressure and shares solid and liquid properties.[131] Both solvents are tunable and can therefore be used for various biomass types.[131] In the case of ionic liquids, the modification of the anion or alkyl substituent(s) of the cation changes solvent properties; adjustments to the temperature and/or pressure of a supercritical fluid tune its properties.[131,134]

Biomass treated with ILs or supercritical fluids demonstrates varied responses in regards to molecular weight. For example, the treatment of bagasse with the IL 1-ethyl-3-methylimidazolium cation yielded over 93% lignin following treatment.[135] However, five ILs used to pre-treat barley straw were identified as unsuitable for lignin removal, due to the minimal changes in the phenolic hydroxyl groups generated by the cleavage of ester linkages and the aromatic skeleton of lignin.[136] Differences in the removal of lignin and the molecular weight of lignin are attributed to solubility. The anion in ILs moderates cellulose solubility, while the cation moderates the solubility of lignin. The combination of the IL 1-ethyl-3-methylimidazolium acetate with the organic solvents dimethylformamide, dimethylsulfoxide, or dimethylacetamide in a 3:7 ratio exhibited less than 300 g mol^{-1} difference in molecular weight of acid insoluble lignins when compared to MWL from corn cob.[137] The complementation of an IL with water, a polar solvent, was 2180 g mol^{-1} greater than MWL from corn cob. Overall, the M_w of lignins isolated from biomass treated with an IL and polar solvent produced the largest molecular weight (M_w 4310 g mol^{-1}). Again, the molecular weights of lignins from the IL and the organic solvents as well as the MWL contained less carbohydrates than the lignins from the IL and aqueous solvent, which increased their relative molecular weights.[137]

The treatment of organosolv lignins with supercritical water (400°C, 1 min) degraded residual lignin molecular weights in either the presence or absence of phenol, as evidenced in the molecular weight distributions of the residual lignins.[85] Low concentrations of acid catalyst, 0.05% HCl, appear to enhance the fragmentation of lignin during sub- and super- critical water pre-treatments in poplar (*Populus albaglandulosa*) wood flour.[102]

Combinatory pre-treatments

Pre-treatments sometimes cross the borders drawn by aforementioned classifications. Physicochemical pre-treatments, such as steam explosion (SE) and ammonia fiber explosion (AFEX), combine elements of physical and chemical pre-treatments. SE produces an explosive decompression in biomass, which generates organic acids from hemicelluloses. Although lignins are removed, they are mostly redistributed throughout the cell fiber after processing.[90] Increases in molecular weight, often attributed to condensation reactions, are sometimes observed following pre-treatment. Acid insoluble lignin recovered from steam exploded wheat straw (*Variety riband*) possessed M_w ranging between 3460 and 3870 g mol^{-1}; however, the addition of an alkaline peroxide post treatment increased the final M_w between 4270 and 4640 g mol^{-1}.[138] The difference in molecular weights was attributed to fewer opportunities for repolymerization in the one stage pre-treatment.[138] Wet oxidation combines SE with an oxidation stage to oxidize lignin and hemicellulose. Although wet oxidation does not remove lignin, it has been found to alter the M_n and M_w in wheat straw and corn stover to values comparable to those generated from hydrothermal pre-treatment.[139]

The molecular weights of transgenic and hybrid lignins

During biorefining, the introduction of modifications to the lignin changes properties that contribute to recalcitrance. For instance, the down-regulation of caffeic acid 3-*O*-methyltransferase decreased acetyl bromide lignin contents between 6.4 and 14.7% in switchgrass (*Panicum virgatum L.*).[140] Several studies have demonstrated that genetic manipulations reduce the degree of polymerization and molecular weight of lignin, relative to an untreated control (Table 15). Eudes *et al.* incorporated 'DP reducers' into *Arabidopsis thaliana*, which produced excessive amounts of C_6C_1 aromatics. The plants possessing the monomer demonstrated a 55% increase in retention times from the SEC chromatograms that represent the smallest lignin fragments when compared to CEL.[141] The overexpression of ferulate 5-hydroxylase gene in hybrid poplar (*Populus tremula* × *alba*) produced excessive amounts of the S monomer, which reduced its degree of polymerization. The relative peak intensities from the residual lignin isolated from hybrid plants increased by ~11% at retention times above 30 min and decreased by ~17% at retention times below 30 min when compared to the chromatogram of the wild type residual lignin.[142]

Genetically modified biomass has also been combined with pre-treatments and demonstrated reduced molecular weights when compared to MWL (Table 16). In two separate studies, *Miscanthus* × *giganteus* that had been treated with ethanol organosolv produced molecular weights of residual lignin that were less than half of the molecular weight of MWL (M_w ~13,800 g mol^{-1}). In addition, Trajano

Table 15. The M_n, M_w, and D of lignins isolated from transgenic and hybrid biomass.

Biomass	Genetic Line	Modification To Lignin Biosynthetic Pathway	M_w (g mol^{-1})	D
Arabidopsis thaliana[144]	Control	–	~4500	~2.2
	Atom1	Replace sinapyl alcohol in lignin with structures that contain 5-hydroxyconiferyl alcohol	~4500	~2.5
Switchgrass (*Panicum virgatum L.*)[145]	Control	–	5300–5500	–
	PvMYB4-OX	Overexpress the transcription factor PvMYB4 to direct carbon deposition away from lignin	4400–4900	–
Eucalypt[146]	Control	–	11,300–15,000	<4
	IP	–	15,000	3.5
	U1 × U2	–	12,900	3.3
	G1 × UGL	–	13,300	3.8
	DG × U2	–	11,300	3.8
Alfalfa (*Medicago sativa*)[15]	Control[a]	–	6000	–
	C3H9a[b]	Downregulate *p*-coumarate 3-hydroxylase to reduce S and G monomers	4000	–
	C3H4a[c]	Downregulate *p*-coumarate 3-hydroxylase to reduce S and G monomers	5500	–
	HCT30a[d]	Downregulate hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase to reduce S and G monomers	4000	–
Populus deltoides × *populus nigra*[147]	MWEL (control A)	2-year-old trees	36,000	4.1
	ASCAD A	Downregulate cinnamyl alcohol dehydrogenase, possessing 30% less activity than the control	26,000	3.8
	ASMOT A	Downregulate O-methyl transferase	32,000	4.2
	ASCAD ×ASMOT	Downregulate cinnamyl alcohol dehydrogenase and O-methyl transferase	28,000	3.8
	MWEL (control B)	6-month-old trees	28,000	5.6
	ASCAD B	Downregulate cinnamyl alcohol dehydrogenase, possessing <10% the activity of the control	16,000	3.8

[a]2% H monomer; [b]48% H monomer; [c]27% H monomer; [d]74% H monomer.
IP: *E. grandis* × *E. urophylla*; U1 × U2: *E. urophylla* × *E. urophylla*; G1 × UGL: *E. grandis* × [*E. urophylla* × *E. globulus*]; DG × U2: [*E. dunnii* × *E. grandis*] × *E. urophylla.*

Table 16. The M_n, M_w, and D of MWL and lignin from pre-treated hybrid biomass.

Biomass	Isolation/Pre-treatment	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Miscanthus × *giganteus*	MWL[148]	8300	13,800	1.6
	Ethanol organosolv[148]	2500	6500	2.6
		3800	6000	1.6
		3100	4300	1.4
		2500	3600	1.4
		2300	3200	1.4
	MWL[18*]	8300	13,700	1.65
	Ethanol organosolv[18*]	4690	7060	1.51
Populus nigra × *Populus maximowiczii*	Ethanol organosolv[149]	783–1515	1105–4191	1.41–3.04

*Values corrected from the original manuscript after a personal discussion with the author.

et al. studied the molecular weights of CEL isolated from *P. trichocarpa* × *P. deltoides* that had undergone hydrothermal batch or flowthrough pre-treatments under several conditions. The CEL recovered from a flowthrough system (20 mL/min, 140°C, 12 min) experienced approximately a 3.5-fold increase in M_w relative to the CEL recovered from batch processing (140°C, 192 min), which was attributed to repolymerization reactions.[143]

Lignin polymerization

Although the former sections have focused on the removal or restructuring of lignin, the polymerization of technical lignins is desirable at times. Technical lignins are lignins that have been reclaimed during pre-treatment and pulping processes; polymerization offers an opportunity to add value and utilization to this byproduct.[150] These polymerization reactions are often catalyzed by oxidative enzymes such as laccases and peroxidases. Mattinen *et al.* revealed the successful polymerization of soluble flax soda lignins, eucalyptus (*Eucalyptus globulus*) dioxane lignin, and spruce (*Picea abies*) EMAL using laccase isolated from the fungus *Trametes hirsuta* without a mediator.[151] Process variables also influence the oxidative polymerization of steam exploded sugar cane bagasse treated with horseradish peroxidase (HRP) or potato polyphenol oxidase (PPO). Conditions that favor polymerization in steam exploded sugar cane bagasse include a basic environment (pH 8–10) and reaction times of less than 4 h for PPO. Under basic conditions, the growing lignin polymers are more soluble than in acidic environments; as a result, the polymer can interact with radicals and continue to grow.[152] Organic syntheses have also been developed to provide a pathway to polymerization that generate an artificial lignin polymer that contains only β-O-4 linkages.[153]

Kraft lignin and its molecular weight

A kraft pulp mill has often been referred to as a first-generation biorefinery that is primarily focused on removing lignin from wood, yielding cellulosic fibers.[154] Historically, the molecular weight of lignin has been studied in this field to better understand kraft pulping.[78,155] More recently, the molecular weight of lignin isolated from kraft pulping liquors has come under renewed interest as a viable resource for polyphenolic polymers to be used as a polymer additive,[156] co-polymer,[19] and as a resource for carbon fiber production.[157,158]

The kraft pulping process generally fragments lignin, thereby decreasing its molecular weight. During kraft pulping, wood chips are added to an aqueous solution of sodium hydroxide and sodium sulfide, which facilitates the cleavage of select bonds in lignin and results in depolymerization of lignin.[21,159] The molecular weights of kraft lignin have been reported within the range of 200 to 200,000 g mol^{-1}.[150] However, the molecular weight of kraft lignin can be variable depending on the type of wood, analysis method, and isolation procedure. Saito *et al.* have studied the properties of low molecular weight (LMW) and high molecular weight (HMW) of softwood kraft lignin (SKL) by methanol fractionation. HMW SKL, which is insoluble in methanol, possessed M_w in the range of 14,900 to 188,000 g mol^{-1} and has been successfully separated from LMW with M_w ranging from 3000 to 86,600 g mol^{-1}, which is soluble in methanol.[160]

Table 17 presents the molecular weights and polydispersities of SKL and hardwood kraft lignin (HKL) from several different samples. For example, Brodin precipitated kraft lignin using LignoBoost followed by ultrafiltration to determine the molecular weight of SKL and HKL. SKL (mixture spruce and pine) and HKL (*Eucalyptus globulus*)

Table 17. The M_n, M_w, and D of softwood and hardwood kraft lignin by size-exclusion chromatography.

Sample/Isolation	M_n (g mol^{-1})	M_w (g mol^{-1})	D
Spruce+Pine[a, 161]	1000	4500	4.5
Spruce+Pine[a,161] (Cut-off 5000 Da)	490	1700	3.5
Spruce+Pine[a,161] (Cut-off 15,000 Da)	580	2900	3.9
Softwood Kraft Lignin Indulin AT[b,73]	1600	6500	4.1
Softwood Kraft Lignin Indulin AT[c,73]	1700	8000	4.7
Curan 100[d,163]	1300	9900	7.6
Curan 100[e,163]	2000	11,000	5.5
Hardwood Kraft Lignin PC-1369[b,73]	1000	3300	3.3
Hardwood Kraft Lignin PC-1369[c,73]	1000	3900	3.9
Birch[78]	7523	19,650	2.7
Eucalyptus globulus[a,161]	530	2300	4.3
Eucalyptus globulus[a,161] (Cut-off 5000 Da)	440	1300	3.0

[a]Lignoboost; [b]acetic anhydride in pyridine; [c]acetic bromide in acetic acid; [d]DMSO/H2O/LiBr; [e]DMAc/LiCl.

were separated using a membrane with molar mass cutoffs of 5000 g mol^{-1} and 15,000 g mol^{-1}, and the M_n, M_w, and D were lower when compared to unfractionated lignin. The results indicated that low molecular weight, low polydispersity, and high purity of SKL and HKL can be isolated according to need. Brodin used these molecular weight fractions to study lignin-based carbon fibers.[161] In order to obtain HMW kraft lignin from LMW, Wells *et al.* have proposed the preparation of HMW kraft lignin using ultrasonication. The molecular weight of the HMW kraft lignin was increased approximately 35% after subjecting kraft lignin to an extensive ultrasonic treatment.[162]

Current applications and future developments

Applications of lignin have been investigated in many research areas, such as a polyol, epoxy resin, feedstock adhesive, and a precursor for carbon fiber. Lignin has been extensively used as polyol for thermoplastic polyester and polyurethane synthesis. In such polymeric product development, the molecular weight of lignin is very important. High molecular weight lignin offers improved performance in materials' mechanical properties through molecular entanglement and enhanced cohesive strength of the matrix.[164,165] For example, waterborne polyurethane when modified with lignin amine at as low as 1% loading experienced enhancements in its performance. Alkali lignin amine with an M_w of ~2000 Da demonstrated improved the mechanical properties by 100%, whereas Kraft lignin amine with an M_w of ~1500 Da improved the mechanical properties by 50%. Hardwood ethanol organosolv lignin exhibited slightly higher molecular weights than that of the hardwood lignin extracted by Kraft process. Kraft lignin possesses a higher number of phenolic and aliphatic hydroxyl groups than the alkali lignin. These lignins when used as low-cost extender for rigid polyurethane foam high molecular weight lignin could exhibit higher loading capacity (30%) without significant deterioration in properties than the low molecular weight counterpart (23%).[166] In all cases depending on the molecular weights, the polyol/isocyanate ratio played a key role on materials properties.

Very low molecular weight lignin isolated from steam exploded rice straw by methanol extraction exhibits 0.67 mmol g^{-1} sample of phenolic hydroxyl group.[167] This value is about 1/13 of the quantity of phenolic hydroxyl groups in bisphenol A and such lignin can be converted to epoxy resin for composite applications. Likewise, low-molecular weight lignin isolated from wheat straw by acetic acid pulping is an ideal feedstock adhesive manufacturing.[39] Compared to many other lignins, Kraft and soda processed lignin exhibit high Mannich reactivity likely due to its low-molecular weight and those are useful for adhesive manufacturing.[168]

The Oak Ridge National Laboratory Carbon Fiber consortium has concentrated on improving the properties of lignin-based carbon fibers in order to obtain a low-cost carbon fiber supply for the automotive industry.[169] Studying the molecular weight properties of lignin is very important for carbon fiber production.[155,161,162] Low molecular weight lignin results in difficulties during melt spinning of carbon fibers by imparting brittleness.[170] Modified high molecular weight properties of lignin have been developed through chemical reactions.[171] Investigating higher molecular weight properties of lignin is also important for value-added lignin-based application such as thermoplastic blends or copolymers.[164,171,172] Because low molecular weights of lignin are related to a smaller shear modulus, fractionation of lignin with methanol and crosslinking with formaldehyde could be another pathway to increase molecular weight properties of lignin in order to improve the quality of the thermoplastic lignin-based

materials. In addition, this novel discovery could lead to new information for petrochemicals applications.[164]

Conclusion

Understanding biomass recalcitrance has been at the forefront of many research studies over the years, specifically with the focus on lignin. The reduction of biomass recalcitrance is related to the molecular weight of lignin. There are numerous combinations of various isolation methods and pre-treatments that allows for the characterization of the M_n, M_w, and D for lignin. MWL, CEL, and EMAL each have different advantages and disadvantages to separating lignin from the biomass. The numerous pre-treatments target the biomass biologically, physically, and/or chemically in order to initially overcome the recalcitrance. And the characterization of the lignin can be accomplished in a number of ways, but the most common used technique is GPC. An ideal pathway to extracting the lignin from biomass currently does not exist, but there are a plethora of studies highlighting procedures that result in high yields of lignin and the corresponding molecular weight.

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