Analytical Techniques in lignin characterization: advances and challenges

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outline

- Lignin biosynthesis
- Lignin heterogeneity
- Lignin structure
- Wet chemistry analytical methods in lignin analysis
- Advanced NMR techniques in functional groups and interunit bondings analysis
- Lignin branching revisited
- Molecular weight distribution
- Molecular weight distribution revisited
- Conclusions
Lignin formation involves:

- biosynthesis of monolignols;
- transport of monolignols to lignifying sites;
- enzymatic radicalization of monolignols; and,
- non-enzymatic coupling of the radical monomers into the growing lignin polymer.

Several aspects of even these simple steps are controversial.
Lignin biosynthesis

- Lignin is a phenyl-propanoid (C9) polyphenol mainly linked by arylglycerol ether bonds between the monomeric phenolic p-coumaryl (H) coniferyl (G) and sinapyl alcohol (S).

Peroxidases-H$_2$O$_2$ systems cause proton abstraction from a given cinnamyl alcohol.
Lignin Interunit Bonding Patterns

- **β-O4, β-5**
  - β-couplings: favoured as monolignol coupling mode and end wise polymerization

- **β-β, β-1**

- **DBDO, 4-O-5, 5-5: 5-couplings:** (favoured as oligomers coupling mode)

- Branching!
Lignin structure

- No repetitive units
- No repetitive bonding patterns

Degree of polymerization and Lignin structure and branching not fully understood
Lack of suitable analytical techniques

Crestini C. et al. *Biomacromolecules*, 2011, 12 (11), 3928–3935
Lignin or Lignins? The heterogeneity of lignins

G-units/S-units in white birch wood

<table>
<thead>
<tr>
<th>Morphological Differentiation</th>
<th>Guaiacyl/Syringyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre, S2-layer</td>
<td>12 : 88</td>
</tr>
<tr>
<td>Vessel, S2-layer</td>
<td>88 : 12</td>
</tr>
<tr>
<td>Ray parenchyma, S-layer</td>
<td>49 : 51</td>
</tr>
<tr>
<td>Middle lamella (fibre-fibre)</td>
<td>91 : 9</td>
</tr>
<tr>
<td>Middle lamella (fibre-vessel)</td>
<td>80 : 20</td>
</tr>
<tr>
<td>Middle lamella (fibre-ray)</td>
<td>100 : 0</td>
</tr>
<tr>
<td>Middle lamella (ray-ray)</td>
<td>88 : 12</td>
</tr>
</tbody>
</table>

Saka and Goring, 1988
Lignin in annual plants and Hardwood species

<table>
<thead>
<tr>
<th>Origin</th>
<th>Lignin content</th>
<th>H:G:S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flax</td>
<td>2.9 (+ 1.6)</td>
<td>57:33:11 (pyrolysis)</td>
</tr>
<tr>
<td>Sisal</td>
<td>10.8 (+ 3.0)</td>
<td>1:20:79 (pyrolysis)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>16.0</td>
<td>5:49:46 (thioacidolysis)</td>
</tr>
<tr>
<td>Rice straw</td>
<td>6.1</td>
<td>15:45:40 (thioacidolysis)</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
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<th>S/G-ratio</th>
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</table>

- Gymnosperms lignin consists almost entirely of G (G-lignin),
- Dicotyledonous angiosperms lignin is a mixture of G and S (G/S lignin)
- Monocotyledonous lignin is a mixture of all three units (GSH-lignin).
Lignin or Lignins?
Isolation Procedures

- Insoluble Lignin Preparations: Klason, Cuoxam, Periodate, etc
  - Destruction of carbohydrates with acids, enzymes, etc: **Lignin structure highly altered.**

- Soluble Lignin Preparations: Bjorkman (MWL), Brauns, etc
  - Lignin is removed from wood (solublized) using different solvents and mechanical techniques. These materials are considered **most representative of native lignin.** Very low yields.
    - Neutral Solvents (Example: MWL, ball milling, dioxane extraction)
    - Acidic Organic Solvents (Example: Alcohol lignin)

- Commercial Lignin Preparations
  - **Highly degraded materials** including Kraft lignin, lignosulfonates, hydrolysis lignin, etc.
Mechano-chemical modification of lignin during milling

Figure 4. Weight-average molecular weight of EMAL isolated from vibratory (■) and ball (△)-milled wood.

Argyropoulos et al. 2006
Lignin Isolation: Commercial lignin preparations

The fractionation of a lignocellulosic raw materials into separate cellulose, hemicellulose and lignin streams.
Kraft lignin

- Kraft pulping is the main chemical pulping process.
- Treatment at 150-180°C with sulfide, sulphydryl and polysulfide at high pH. Solubilised lignin is localized in the spent pulping liquor (“black liquor”) along with most of the wood’s hemicellulose.
- Kraft lignin may be recovered from the black liquor by precipitation lowering the pH.
Kraft lignin

- Kraft lignin is soluble in alkali and in strongly polar organic solvents. Its number average molecular weight (Mn) is generally between 1000 and 3000, but exhibits a polydispersity typically between 2 and 4.

- Kraft pulping of wood constitutes potentially the source of the largest amount of lignin for the biorefinery.
Lignosulfonate lignin

- Sulfite pulping is carried out between pH 2-12.
- Use calcium and/or magnesium as the counterion.
- Higher pH sulfite pulping is generally done with sodium or ammonium counterions.
- Sulfite lignin contains considerable sulfur in the form of sulfonate groups present in the aliphatic side chains.
Lignosulfonate Lignin

- Sulfite lignin generally is soluble throughout the entire pH range.
- Recovery of sulfite lignin (lignosulfonate) is commonly done from waste pulping liquor concentrate after stripping and recovery of the sulfur.
- Mn values of **5,000-20,000** are common.
- polydispersity is higher than kraft (4 to 9)
- sulfur content (3% to 8%).
Organosolv lignin

- Organosolv pulping is a general term for the separation of wood components through treatment with organic solvents.

- The most well known is the Allcel process which uses ethanol or ethanol-water as solvent.

- Organosolv lignin can be separated from the pulping solvents by solvent removal and recovery or a combination of precipitation with water accompanied by distillation to recover solvent.

- Organosolv lignin is insoluble in water between pH 2 and 7 but will dissolve in alkali and many polar organic solvents.

- Mn values are typically less than 1000.

- Polydispersity ranges from 2.4 to 6.4.
Steam explosion consists in biomass impregnation with steam (180-230°C) under high pressure (200-500 psig) at short contact time (1-20 min) followed by rapid pressure release.

The steam explosion process allows release of individual biomass components and the process has generally been used as a method for preparing cellulose pulp.

Alkali washing or extraction with organic solvents allows recovery of hardwood lignins up to 90%.

Steam explosion lignin shows a lower molecular weight and higher solubility in organic solvents than kraft lignin.
Lignin Structure
Classical Analytical Procedures

- Since lignin is a polydisperse polymer with no extended sequences of regularly repeating units, its composition is generally characterized by the relative abundance of H/G/S units and by the distribution of interunit linkages in the polymer.

- Functional groups
  - Wet Chemistry techniques
  - Spectroscopic-spectrometric techniques

- Linkages
  - Degradation Studies
  - NMR
  - Biosynthesis studies
Classical analytical degradative methods

- Permanganate oxidation, nitrobenzene oxidation, GC-MS pyrolysis, thioacidolysis and derivatization followed by reductive cleavage (DFRC) are degradative methods that reveal the **H/G/S composition of the lignin polymer**.

- All these methods **liberate only a fraction** of the polymer for analysis. They are based on the cleavage of lignin backbone and analysis of the fragments obtained, and provide partial data due to the specificity of the treatments.
Lignin Structure Elucidation Studies

Nitrobenzene Oxidation

Rapid semi-qualitative indication of G/S/H units
- Not too informative
- Very laborious/lengthy/experimentally intensive
- Prone to errors
Total ion chromatogram of nitrobenzene oxidation mixture from milled bamboo lignin (MBL)

1 p-Hydroxybenzadehyde
2 Vanillin
3 Syringaldehyde
In permanganate oxidation the side-chains are degraded to carboxyl groups attached to the aromatic ring.

The structure of the products reveal the substitution pattern of the aromatic rings.

Only looks at phenolic unit

Very laborious/lengthy/experimentally intensive

Prone to errors

- the low yields of degradation acids make it difficult to estimate the quantitative distribution of structural units
During thioacidolysis arylglycerol-\(\beta\)-aryl ethers are selectively cleaved by a treatment with boron trifluoride etherate and ethanthiol. Monomeric products substituted with thioethyl groups are formed and can be analysed by gas chromatography. Dimeric products can be analyzed after removal of sulfur substituents by reduction with Raney-Nickel.

An important limitation of thioacidolysis is that it can only detect structural units bound by arylglycerol-\(\beta\)-ether bonds.

*Not a simple technique, Requiring a malodorous reagent, Potential incomplete cleavage*
Thioacidolysis – Mechanism

$\text{Et}_2\text{O}-\text{BF}_3 + \text{Et}_2\text{O}-\text{BF}_3 \rightarrow \text{Et}_2\text{O}-\text{BF}_3 + \text{Et}_2\text{O}-\text{BF}_3$

$\text{R}^1=\text{Ar} \quad \text{R}^2=\text{H} \text{ or } \text{Ar}$
$\text{R}^3 \text{ or } \text{R}^5=\text{H}, \text{OMe} \quad \text{R}^4=\text{H} \text{ or } \text{Alkyl}$
Thioacidolysis
Selective ether cleavage by the DFRC method

is an other selective β-arylether cleavage protocol
• bromination of the benzylic positions and acetylation of hydroxyl groups
• reductive cleavage of the brominated intermediate and acetylation.

Quantification of products is carried out by gas chromatography
presence of elemental bromine, existence of intact β-O-4 interunit linkages and
high average relative molecular weight of the treated lignin samples indicates
that the DFRC method does not completely or efficiently degrade the lignin polymer
GC of DFRC Monomers

A

16s

16c

B

17s

17c

C

18s

18c

D

17s

17c

18s

18c

(Min)
GC of Lignins

Pine Lignin

Willow Lignin
Significance of degradative analysis methods

- A wet chemistry method that allows the contemporary identification and quantification of all the interunit bondings in lignin is NOT available.

- High degree of uncertainty
  - Uncertainty bound to the completeness of lignin degradation processes
  - Occurrence of eventual side reactions
  - Different analytical methods applied to same lignin samples provide significantly different results that are not directly comparable.

This problem, coupled with the extreme heterogeneity of lignin preparations and lignin chemical structure among wood species, morphology and maturation degree makes to date the quantitative structural characterization of lignin is still an open debate.
The Lignin Paradigm

Lignin is a random, three-dimensional network polymer

- High branching degree
- High molecular weight

Softwood lignin models designed by Nimz (a) and Brunow (b)
The Numbers of Lignins

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<td>Gutierrez et al, 2007</td>
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</table>
The Numbers of Lignins

<table>
<thead>
<tr>
<th>Linkage type</th>
<th>Dimer structure</th>
<th>Percent of total linkages</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Softwood</td>
</tr>
<tr>
<td>β-O-4'</td>
<td>Arylglycerol-β-aryl ether</td>
<td>50</td>
</tr>
<tr>
<td>α-O-4'</td>
<td>Noncyclic benzyl aryl ether</td>
<td>2-8</td>
</tr>
<tr>
<td>β-5'</td>
<td>Phenylcoumaran</td>
<td>9-12</td>
</tr>
<tr>
<td>5-5'</td>
<td>Biphenyl</td>
<td>10-11</td>
</tr>
<tr>
<td>4-O-5'</td>
<td>Diaryl ether</td>
<td>4</td>
</tr>
<tr>
<td>β-1'</td>
<td>1,2-Diaryl propane</td>
<td>7</td>
</tr>
<tr>
<td>β–β'</td>
<td>Pinoresinol/lignan type</td>
<td>2</td>
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<table>
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<tr>
<th>Functional group</th>
<th>Hardwood</th>
<th>Softwood</th>
</tr>
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<tbody>
<tr>
<td>Methoxy</td>
<td>1,5/C9</td>
<td>0,95/C9</td>
</tr>
<tr>
<td>Phenolic OH</td>
<td>10-15/100C9</td>
<td>15-30/100C9</td>
</tr>
</tbody>
</table>
NMR aided lignins characterization

- $^{13}$C-NMR of lignins or acetylated lignins
  - Qualitative assignments
    - Interunit bondings
  - Quantitative data
    - Aliphatic and phenolic OH groups
    - Methoxy groups

- HSQC of lignins or acetylated lignins
  - Qualitative/Quantitative assignments
    - Interunit bondings

- $^{31}$P-NMR of phosphorylated lignins
  - Qualitative and Quantitative assignments of aliphatic and phenolic OH groups, guaiacyl, siringyl, condensed OH groups, carboxylic acids
Lignin Characterisation: $^1$H-NMR
Lignin Characterisation: $^{13}\text{C}-\text{NMR}$

Zhang et al. 2006

Quantitative $^{31}\text{C}-\text{NMR}$ softwood and hardwood MWL
$^{13}$C-NMR quantitative issue

- Primary and secondary aliphatic and phenolic OH groups
  - 169.9-171, 169.5 and 168.6 ppm
- Methoxy groups
- Internal standard: trioxane
- Quantitative methoxy or hydroxy groups analysis for indirect quantification
$^{13}$C-NMR qualitative issue: DEPT
NMR Lignin trifluoromethylation

Figure 1. Trifluoromethylation of carbonyl-containing (including quinones) lignin-like model compounds.

Figure 8. $^{19}$F NMR spectra of trifluoromethylated dioxane lignin (A), after Dakin reaction (B) and reduction (C) with sodium hydrosulfite.
Lignin characterization: $^{31}$PNMR

In the Case of Typical Lignins

1. $\sim 40$ mg Lignin accurately weight
2. 500 $\mu$L Py-D$_5$/CDCl$_3$ (1.6/1, V/V)
3. 200 $\mu$L I.S. solution
4. 50 $\mu$L Cr(acac)$_3$ as relaxation agent
5. 100 $\mu$L Phosphitylation reagent

$I.S.$ solution = 50.0 mmol/L in Py-D$_5$/CDCl$_3$ (1.6/1, V/V))
$Cr(acac)_3$ solution = 11.4 mg/mL in Py-D$_5$/CDCl$_3$ (1.6/1, V/V))
Lignin Characterization: $^{31}$P-NMR

R = H, OCH$_3$, Lignin
1: $R'$ = H
2: $R'$ = CH$_3$

Journal of Agricultural and Food Chemistry, 43(6), 1538-1544, 1995
N-Hydroxynaphthalimide

R = R' = H

COST ACTION E41: Round Robin
$^{13}\text{C}$- vs $^{31}\text{P}$-NMR

Average data from nine experiments

**Spruce EMAL**
- OCH$_3$: 3.50 mmole/g (Average)
- Phenolic OH: 0.99, 0.84, 0.84 mmole/g
- Aliphatic OH: 4.59, 4.69, 4.72 mmole/g
- Total OH: 5.58 mmole/g

**Eucaliptus EMAL**
- OCH$_3$: 7.8 mmole/g (Average)
- Phenolic OH: 1.01, 0.77, 0.82 mmole/g
- Aliphatic OH: 6.46, 6.61, 6.58 mmole/g
- Total OH: 7.47 mmole/g

**EMAL**
- $^{13}$C-NMR ES
- $^{13}$C-NMR IS
- $^{31}$P-NMR
**$^{31}$P-NMR after DFRC**

- Coupled to the spread of advanced NMR techniques, during the past decade lignin structural inquiries have been greatly facilitated by the development of various degradative protocols, such as DFRC (Derivatization Followed by Reductive Cleavage) which efficiently cleaves the $\beta$-aryl ethers in lignins.

- The combination of DFRC with quantitative $^{31}$P NMR was shown to have significant potential for the determination of arylglycerol-$\alpha$-aryl ether and other linkage.
31P-NMR after DFRC

1. Acetylation of free phenolic groups (terminal phenolics)

2. Selective reductive cleavage of β-aryl ether bondings with release of phenolic lignin fragments

3. 31P-NMR of the released phenolic fragments

Lignin Characterisation: 2D-NMR
TOCSY
HSQC

• (2D) $^{13}$C-$^1$H-correlated (HSQC, HMQC) spectroscopy, which combines the sensitivity of $^1$H NMR with the higher resolution of $^{13}$C NMR,

• best method to reveal the different lignin units and the interunit bonding patterns

• use as a valid analytical technique in the analysis of complex samples

• The QQ-HSQC NMR pulse sequence can be successfully applied to lignin quantitative structural elucidation. The standard deviations of the results were found ranging from 0.01 to 2 bondings per 100 C$_9$ units
Lignin characterization: Quantitative HSQC

Signals were normalised to the $G_2$ C-H taken as measure of the aromatic units (internal standard).

QQ-HSQC: Hardwood Milled Wood Lignin

S/G ratio determination

- S-β-4
- β-O-4
- β-β
- β-5
- β-1

<table>
<thead>
<tr>
<th>Lignin interunit bonding</th>
<th>Number per 100 C₉ units</th>
<th>Zhang[32]</th>
<th>Capanema[33]</th>
<th>Robert[34]</th>
<th>Adler[13,35]</th>
</tr>
</thead>
<tbody>
<tr>
<td>QQ-HSQC</td>
<td>NMR methods*</td>
<td>Wet chemistry method*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β – O-4</td>
<td>44.7 ± 0.9</td>
<td>40–43</td>
<td>36–45</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>β - 5</td>
<td>10.6 ± 2.0</td>
<td>10–12</td>
<td>9</td>
<td>10</td>
<td>9–12</td>
</tr>
<tr>
<td>β – β</td>
<td>3.19 ± 0.01</td>
<td>3.5</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>β - 1</td>
<td>1.25 ± 0.34</td>
<td>2</td>
<td>1</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>5–5’-O-4</td>
<td>3.07 ± 0.05</td>
<td>5</td>
<td>7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SD</td>
<td>0.24 ± 0.05</td>
<td>1.2–2</td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Paradigm: Lignin is a network polymer

Lignin Branching revisited

- Phenolic 4-O-5, DBDO or 5-5 Terminal units: no branching!

- 4-O-5 $^{31}$P-NMR 142ppm
- 5-5 $^{31}$P-NMR 141ppm
- DBDO QQ-HSQC
Lignin Branching

- **Non-phenolic** DBDO, 4-O-5 or 5-5 units: Branching!

- Evaluation of lignin branching consists in quantification of DBDO, non-phenolic 5-5 and 4-O-5 lignin subunits
Lignin Branching

- Non-phenolic DBDO are absent in MWL (Ralph et al. ISWFPC 2009) (HSQC)
- non-phenolic 4-O-5 are absent in MWL (Crestini et al. 2011) (\(^{31}\text{P}-\text{NMR}\) after DFRC)
- all etherified 5-5 bonds are dibenzodioxocins (Crestini et al. 2011) (QQHSQC+ \(^{31}\text{P}-\text{NMR}\) after DFRC)

The Lignin Paradigm revisited:
Branching is not significantly present in MWL
Molecular Weight Distribution in Lignin

- GPC
- MALDI-ToF MS
- Vapour osmometry
- Isopiestic methods
- Cryoscopy
- Ultrafiltration
- Light scattering

- Different methods give not reproducible data
- Determinations based on colligative properties yield Mn from 500 to 5000 D. (DP ranging from 3 to 25).
- It was clear also in early studies that association phenomena were operative
- GPC and light scattering provide higher values
- Supramolecular association
- Dependence on the solvent
- Time dependance
- Lack of standards
Mass spectrometry

- ESI-MS.
  - study of *in vitro* model of lignin biosynthesis, polymerization reaction course of coniferyl alcohol;
  - molecular weight determination and structural characterization of a series of technical lignins;
  - investigation of lignin oligomers obtained after thioacidolysis of lignin model compounds and Milled Wood Lignin (MWL).

- MALDI-MS
  - molecular weight determination of Milled Wood Lignin (MWL);
  - molecular weight determination technical lignins
  - study of the *in vitro* model of lignin polymerization reaction course and the *in vitro* copolymerization with carbohydrates.
MALDI Mass spectrum of birch MWL

Fine Structure of the MALDI mass spectrum of birch MWL

$\Delta m=190-210\text{Da}$
Phenylpropane unit

Hyperfine Structure of the MALDI mass spectrum of birch MWL

$\Delta m=30\text{Da}$
Methoxy group
Fine structure of the MALDI mass spectrum of conifer MWL

$\Delta m=190\text{Da}$
Guaiacyl Propane Unit

- Up to nonamer (birch) or decamer (conifer)
- Many different oligomers with the same degree of oligomerization are present in lignin
MALDI spectra of the reaction mixtures obtained by horseradish Peroxidase/H$_2$O$_2$ dehydrogenation of coniferyl alcohol

Paradigm: Lignin is a network polymer
Lignin MW revisited

- MALDI ToF MS analysis shows interesting peaks also at oligomeric level and gives values comparable to vapor osmometry determination of low Mn.

- Although GPC gives results depending on a number of different factors as for example
  - Solvent
  - Salt addition
  - pH
  - Acetylation degree

- It is currently the most widespread analytical method currently in use for Mn lignin determinations due to its ease of use and low time consume in the experimental determinations.
End-Groups Titration

- An analytical method suitable for determination of DP of oligomers and small polymers is the end groups titration.
- Completely independent on supramolecular association phenomena.
- Unequivocal determination of average DP and $M_n$ values.
- NMR spectroscopy is a technique that allows to determine the average degree of polymerization of a polymer avoiding interferences due to supramolecular aggregation, provided the knowledge of monomer formula weight and the possibility to quantitatively integrate the amount of end groups and monomers of the polymer studied.
Lignin MW revisited: End Group Titration vs GPC

<table>
<thead>
<tr>
<th>lignin sample</th>
<th>Mn (GPC)</th>
<th>Mn (NMR-end group titration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS-MWL</td>
<td>14200</td>
<td>1850</td>
</tr>
<tr>
<td>NS-EMAL</td>
<td>7300</td>
<td>1430</td>
</tr>
<tr>
<td>Beech-MWL</td>
<td>9650</td>
<td>2650</td>
</tr>
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Conclusions: Advances in lignin structural characterization

- Lignin is diverse and heterogeneous
- Degradative analytical techniques are lengthy and complicate
  - Qualitative or semi quantitative
  - Side reactions and/or incomplete reactions
  - Possible artifacts
- NMR & Spectrometric Techniques
  - More sensitive & reliable
  - Quantitative HSQC techniques are the most diagnostic and accurate analytical tools to investigate lignin structure
Conclusions: lignin challenges

- The unequivocal determination of the distribution of MW in lignins is still a challenging task
- ALL paradigms, based on older experimental indirect evidences should be revisited
  - Lignin branching
  - Lignin polymeric nature
- The role of supramolecular assembly has to be clarified
- The nature of lignin carbohydrate linkages and occurrence is not completely clear
- The isolation processes affect the lignin structure
Lignin is a highly aggregated linear oligomer

- No repetitive units
- No repetitive bonding patterns

Crestini C. et al. *Biomacromolecules*, 2011, 12 (11), 3928–3935
THANK YOU FOR YOUR ATTENTION!