The Flatness of Lignosulfonate Macromolecules as Demonstrated by Electron Microscopy

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Synopsis

A high molecular weight lignosulfonate was examined in the electron microscope using three different methods of preparation. In all cases, the lignosulfonate macromolecules appeared on the photomicrographs as spots about 10 nm in diameter. From the molecular weight and the diameter of the spots, it was deduced that the lignosulfonate macromolecules were irregularly shaped disks lying flat on the carbon film with an average thickness of about 2 nm. This conformation was consistent with the lamellar structure previously proposed for the cell wall of wood.

INTRODUCTION

Fourteen years ago electron micrographs of lignosulfonate macromolecules were published.[1] The method used to obtain contrast was negative staining. One of the early photomicrographs of a high molecular weight sample is reproduced in Figure 1. The lignosulfonate macromolecules appear to be spherical in shape and to cover a wide range of sizes.

The spherical shape of the macromolecule was expected since studies of the solution properties of several types of lignin[2] had indicated that the slopes of the logarithmic plots of molecular weight against the intrinsic viscosity, the diffusion coefficient, or the sedimentation constant lay between the values expected for an Einstein sphere and a nonfree-draining random coil. A microgel model was proposed which, hydrodynamically, would approximate to a solvent-swollen sphere. Thus, the circular shapes seen in Figure 1 were taken as support for the spherical conformation of the macromolecule.

Recently, however, an alternative conformation has been proposed for the lignin macromolecule.[3] Soluble lignins are envisaged as being made up of flexible, disk-like molecules having various shapes and sizes but all with approximately the same thickness of 2 nm. Two lines of reasoning have led to this new proposal. Firstly, Luner and Kempf[4] have found that a variety of soluble lignins when spread on a liquid surface give monolayers approximately 1.8 nm thick, regardless of the molecular weight of the lignin fraction used. If the molecules were spherical on the surface, the thickness of the monolayer should increase with the molecular weight.[3]

The second line of reasoning arises from the recently proposed lamellar structure for the secondary wall of the wood cell shown in Figure 2.[5] In it, the lignin is sandwiched between interrupted lamellae of polysaccharide, with the thickness of the lignin lamellae being about 2 nm. During delignification, the lignin lamellae are broken up and the lignin macromolecules produced are dis-

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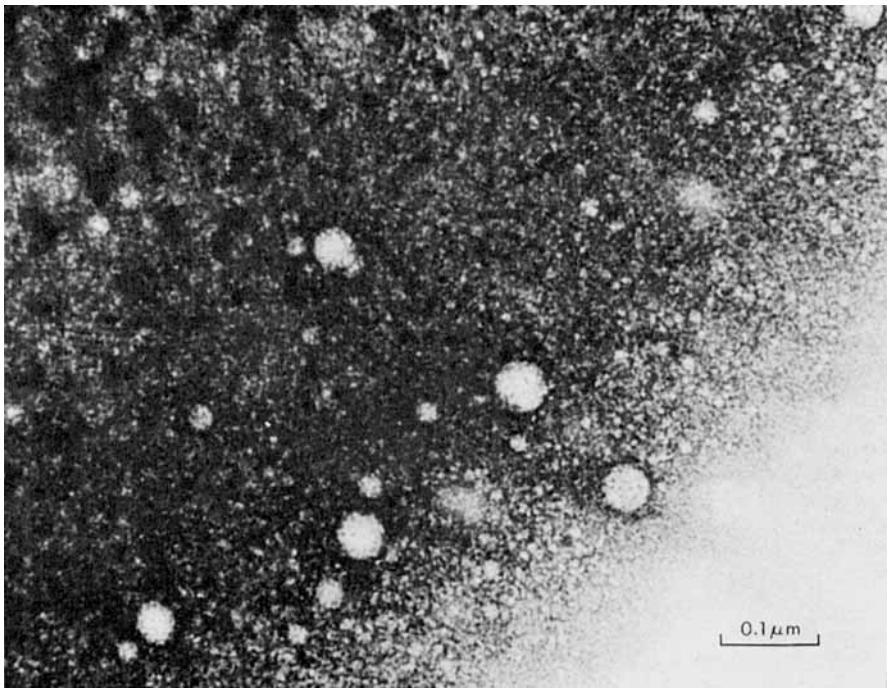

Fig. 1. Electron micrograph of a high molecular weight lignosulfonate negatively stained with phosphotungstate.[1]

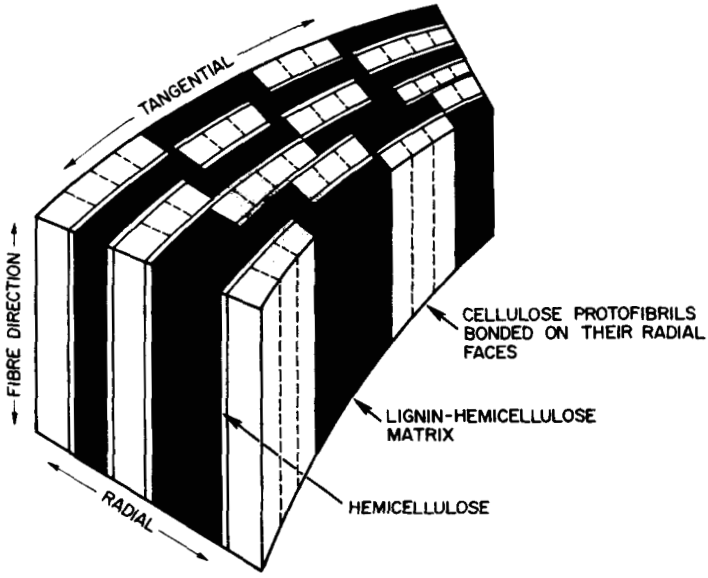

Fig. 2. Pictorial representation of the proposed interrupted lamella model for the ultrastructural arrangement of lignin and polysaccharide in the wood cell wall.[5]

solved in the form of disk-like fragments as shown in Figure 3.[3] Such molecules would be expected to be flexible and to assume an approximately spherical conformation in solution. Moreover, their hydrodynamic behavior will lie be-

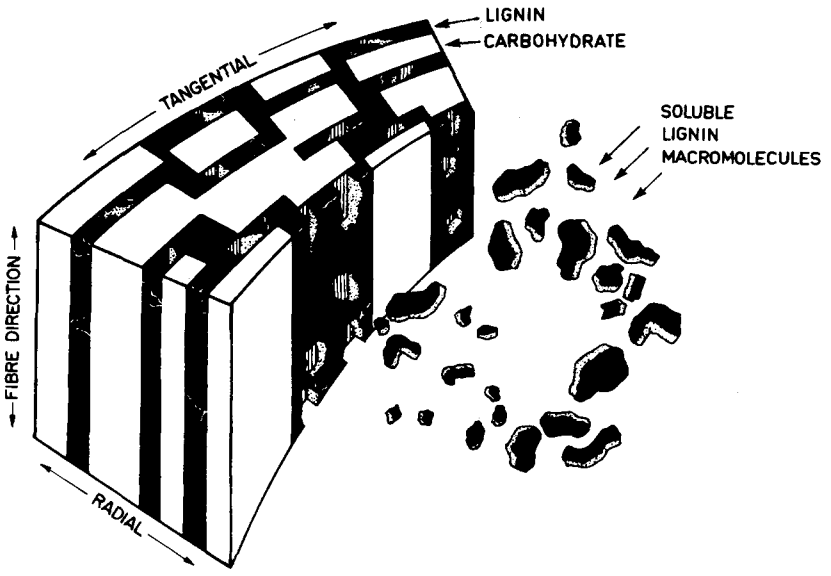

Fig. 3. Pictorial representation of the breakdown and solution of lignin during chemical pulping.[3]

tween that of an Einstein sphere and a linear nonfree-draining random coil. However, when spread out in a monolayer or adsorbed onto a surface, the lignin macromolecules would be expected to assume a flat configuration in keeping with the lamellae structure from which they were originally derived.

If the picture described above is correct, it should be possible to deposit lignin macromolecules onto a carbon film for electron microscopy in a flat conformation. Indeed, the circular shapes photographed in Figure 1 may have corresponded to a flat disk-like conformation rather than to a spherical one. In order to test this concept, it was decided to reinvestigate the electron microscopy of lignosulfonate macromolecules in a manner more quantitative than was previously done. By a comparison of the dimensions of the image with the weight of the molecule, the thickness of molecule on the carbon film was calculated and compared with the thickness determined previously by Luner and Kempf from monolayer experiments.[4]

EXPERIMENTAL

The sample of lignin used was a sodium lignosulfonate, fraction CAS-6, described in a previous publication.[6] It had a sulfur content of 5.7%, a methoxyl content (on a nonsulfur basis) of 14.0%, and a weight-average molecular weight of 143,000.

A 0.01% solution of the lignosulfonate was made up in distilled water and centrifuged at 2600 g for 30 min in order to remove dirt and colloidal debris. A drop of solution was placed on a carbon-coated copper grid and allowed to dry down under dust-free conditions. Prior to the addition of the lignosulfonate solution, the grids were treated in a glow discharge in air at 0.3 torr in order to render them hydrophilic and thus to ensure spreading of the lignosulfonate macromolecules on the surface of the carbon.

The grids carrying the specimen were either negatively stained or metal shadowed. For negative staining, the grids were treated with a fresh solution of 2% phosphotungstic acid brought to pH 5 with 1% KOH. After 5 min of contact with the stain, the excess was removed and the grid allowed to dry. As short an interval as possible was allowed to elapse between the preparation of the negatively stained grids and their examination in the microscope. For shadowing, an alloy of tantalum and tungsten was sputtered onto the grids. The shadowing angle was 30°, and the pressure was 2×10^{-6} torr.

In addition to negative staining and metal shadowing, positive staining was used in specimen preparation. The sodium cation was exchanged to cesium by prolonged dialysis of a 0.1% solution of the sodium lignosulfonate against 20m*M* aqueous CsBr. Excess CsBr was then removed by dialysis against distilled and deionized water for 24 hr. The solution of cesium lignosulfonate was then diluted to 0.01%, centrifuged, and spread onto grids covered with an ultrathin film of carbon. Thin films were supported on thicker holey films which were first coated with a thin layer of gold.

The specimens were then examined in a Philips EM 300 electron microscope with a voltage of 80 kV. Bright-field illumination was used for the specimens prepared by negative staining and metal shadowing; the positively stained specimens were examined by dark-field illumination using the cone illumination technique.

RESULTS AND DISCUSSION

Photomicrographs of the lignosulfonate prepared by the three methods are shown in Figure 4. In all three the same general appearance is evident; the lignosulfonate shows up as irregularly shaped spots of various sizes. The individual spots are seen most clearly in the negatively stained specimen (A). However, the photomicrograph of the metal-shadowed specimen (B) gives essentially the same effect. The spots are least clearly differentiated in the photomicrograph of the positively stained specimen (C). However, close examination reveals that the black areas are indeed irregularly shaped spots a little smaller than those shown by negative staining and metal shadowing. The similarity of the images

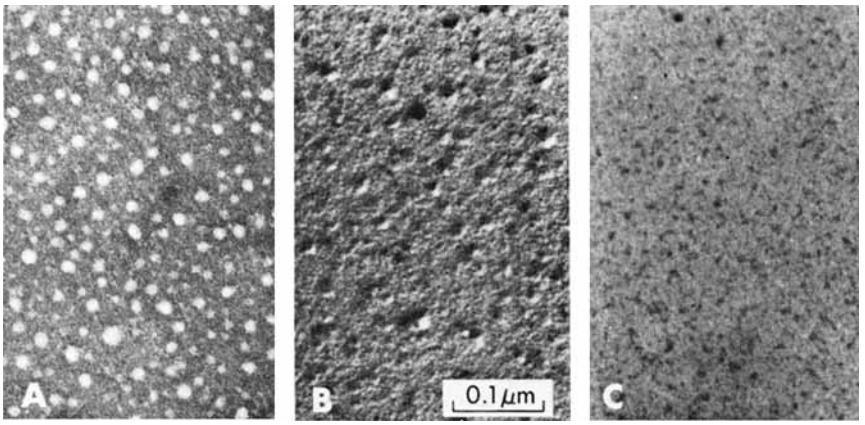

Fig. 4. Photomicrographs of lignosulfonate prepared by negative staining (bright field) (A), metal shadowing (bright field) (B), and positive staining (dark field: reverse print) (C).

given by the three methods of preparation suggests that the spots correspond to individual lignosulfonate macromolecules.

The size of the spots on the photomicrographs was measured on prints at a magnification of ×195,000. A square 30 mm × 30 mm was drawn on the print, and the diameters of all the spots in the square were measured in two orthogonal directions. There were between 25 and 50 spots in each square. The root mean square value of this dimension was then calculated. Four such sets of measurements were made on four different 900 mm^2 areas; a mean value was calculated from the four results for each method of specimen preparation.

The results of the measurements are shown in Table I. For the three types of specimen preparation, the spot diameter was approximately the same at 2 mm. The spots measured on the photomicrographs of the stained specimens are somewhat smaller than those measured on the photomicrographs of the metal-shadowed specimen. For the same feature, metal shadowing would be expected to give a larger image than either negative or positive staining. Also noteworthy is the larger error in the measurements for the cesium-stained specimen (C in Fig. 4) because of the smaller contrast of the image.

If each spot on the photomicrograph corresponds to an individual lignin macromolecule on the specimen, we can compute the root mean square diameter D of the macromolecule from the spot size and the magnification. As shown in Table I, the value of D is about 10 nm for the three methods of specimen preparation. If we assume a flat, disk-like shape for the lignosulfonate molecules on the carbon film, the thickness T of the molecule can be computed from

$$T = 4M/\pi D^2 N\rho \tag{1}$$

in which M is the molecular weight, N is Avogadro's number, and ρ is the density of the lignosulfonate. The partial specific volume of sodium lignosulfonate has been reported[7] as 0.614 ml/g, which corresponds to a density of 1.63 g/ml. Substituting this value for ρ in eq. (1), as well as 143,000 for M, we can calculate a value of T which corresponds to each value of D given in Table I.

As shown in the last column of Table I, the thickness of the lignosulfonate macromolecule calculated by means of eq. (1) was between 1.5 and 2.1 nm depending on the method of specimen preparation used. These results may be compared with the value of 1.8 nm given by the data of Luner and Kempf.[4] In view of the completely different experimental methods used in the two investigations, the agreement is very good and provides strong support for a flat conformation for lignin macromolecules when they are spread out on a carbon film.

It should be noted that the words "disk-like" and "flat" are used in a statistical sense only. A true disk is circular in a plane perpendicular to the principal axis,

TABLE I
Dimensions of Lignosulfonate Macromolecules as Measured on Electron Micrographs

Specimen preparation	Diameter of spot on micrograph, mm	D, nm	T, nm
Negative staining	1.89 (σ = ±0.10)	9.7	2.0
Shadowing	2.17 (σ = ±0.05)	11.1	1.5
Positive staining	1.82 (σ = ±0.27)	9.3	2.1

whereas the lignin macromolecules are revealed by the electron micrographs to be irregular in shape but roughly circular. Also it is likely that the thickness T will change from molecule to molecule and may vary within an individual macromolecule. Such variations will arise as a result of the nonuniformity of the lignin lamellae in the cell wall of the wood. Nevertheless, in spite of these reservations, it seems likely that lignin macromolecules are flat and disk-like and thus reflect the morphological structure of the wood from which they are derived.

Finally, it may be of some interest to speculate on the origin of the lamellar structure of the lignin in the secondary wall. Wardrop and Bland[8] have shown that lignification of the cell wall occurs after the polysaccharide portion of the tracheid has been laid down. Siegel[9] found that eugenol could be polymerized with peroxidase on the surface of filter paper to give a lignin-like product. If the filter paper was absent, the polymerization was not as effective. Perhaps the polysaccharide surface plays a role in the polymerization of lignin monomers in the secondary wall. One envisages the phenyl propane units diffusing into the wall and forming layers of only a few monomer molecules thick. Polymerization then takes place to give the lamellar structure shown in Figure 2. Further work on morphological aspects of the biogenesis of lignin is required to support or disprove these speculations.

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