Fractal Analysis of STM Images of Photochemical Polymer of Coniferyl Alcohol

K. Radotić[1], M. Tasić[2], M. Jeremić[3], Z. Budimlija[2], J. Simić-Krstić[4], A. Polzović[5] and Z. Božović[1]

1 *Centre for Multidisciplinary Studies, University of Belgrade, POB 373, 11000 Belgrade, Yugoslavia*
2 *Department of Forensic Medicine, Faculty of Medicine, University of Novi Sad, Novi Sad, Yugoslavia*
3 *Faculty of Physical Chemistry, University of Belgrade, Belgrade, Yugoslavia*
4 *Laboratory for Molecular Machines, Mechanical Faculty, University of Belgrade, Belgrade, Yugoslavia*
5 *Department of Anatomy, Faculty of Medicine, University of Novi Sad, Novi Sad, Yugoslavia*

Abstract. Fractal analysis was applied to images of photochemical lignin polymer obtained using scanning tunneling microscope. We studied the polymer obtained *in vitro* by ionic mechanism through UV radiation – induced polymerization. The analysis showed the regularity of the lignin-like polymer at different levels of organization. At the 95 % confidence level, there was no significant difference in the fractal dimension between images representing different organizational levels of photochemical lignin. That means that lignin produced in *in vitro* conditions by photochemical mechanism of synthesis, has a fractal structural organization. The obtained values of the fractal dimension are in good agreement with the theoretically predicted value for the polyaddition and polycondensation mechanism of polymerization, known as the bulk model.

Key words: Fractals — Lignin — Photochemical polymerization — Scanning tunneling microscopy

Introduction

The concept of "fractal geometry", introduced in science by Mandelbrot (1983), has been used in various scientific disciplines during several recent years. Its principle is based on the fact that most shapes in nature cannot be described well enough

---

Correspondence to Dr Ksenija Radotić, Centre for Multidisciplinary Studies, University of Belgrade, 29 Novembra 142, 11000 Belgrade, Yugoslavia
E-mail `xenia@ibiss.bg.ac.yu`

using ideal constructions of Euclidean geometry, because they are relatively "irregular". Since an important characteristic of fractal geometry is the property of "self-similarity", in statistical sense, fractal structure should have a constant fractal dimension as the image is viewed at different levels of magnification, i.e. different range of scale (Gluck 1986; Falconer 1990). These facts were used to develop methods for "complexity measurements" of different natural shapes (Glenny et al. 1991; Cross et al. 1994).

In a recent study, Rabouille et al. (1992) observed fractal pattern in glycoprotein, protein and polysaccharide macromolecules, suggesting that fractal patterning is a general property of biological polymers. Also, a correlation was demonstrated between fractal organization and biological activity of macromolecules, with possible practical applications of this approach (Ramakrishnan and Sadana 1999). Results of fractal analysis of biological macromolecules may be of importance answering the question whether there is a relationship between geometry of macromolecular organization and certain biological functions (Yamane et al. 1998).

The scaling approach is the most suitable method for quantitative and qualitative studies of the networking polymers, which also lignin belongs to, at the supramolecular level (Kokorevich et al. 1989). Lignin is a structural polymer of the plant cell wall, compartment of the plant cell consisting of a highly organized network of polymers embedded in a solution containing several enzymes, organic and inorganic substances. Free-radical polymerization of phenolic alcohols (coniferyl, *p*-coumaryl and synapyl alcohols) catalyzed by different cell wall peroxidases is adopted as the mechanism of lignin synthesis in the cell wall (Lewis and Yamamoto 1990). However, much remains unknown about lignin formation, structure and bonding environment in the cell wall, because of the great heterogeneity of polymers at the subcellular level and lack of method for isolation of lignin in unaltered form.

It is widely accepted that lignin has protective role in the plant cell against different kinds of stress (Lewis and Yamamoto 1990). On the other hand, photochemical activity of lignin and its precursors is also known (Freudenberg and Dietrich 1953; Leary 1971; Lewis et al. 1989; Radotić et al. 1997, 1998a). Since the cell wall is the first target place of light action on the cell, the knowledge of the photochemistry of lignin and its precursors is important for the understanding of the biochemistry of the cell wall. In our previous papers we studied UV light - induced photochemical reactions of coniferyl alcohol (Radotić et al. 1997), as well as the structure of the polymer obtained as the end product of the polymerization (Radotić et al. 1998a). We compared the structure of the photochemical polymer with that of the more known enzymic polymer. Such *in vitro* studies prove helpful in revealing the mechanisms of lignin synthesis and its structural organization, in particular the regularity of the polymer structure.

There are a few studies of lignin fractal properties in solution. Lignin fractal dimension was studied on the basis of hydrodynamic data and theoretical considerations (Shumilin and Zhurbilo 1988; Kokorevich et al. 1989; Karmanov and Monakov 1995). In our recent study, we determined the fractal dimension of enzymic lignin obtained by *in vitro* synthesis (Radotić et al. 2000), using STM images of the polymer, obtained in our previous work (Radotić et al. 1994). The fractal analysis confirmed the structural regularity of the enzymic polymer (Radotić et al. 2000). In the present study, we determined the fractal dimension of photochemical lignin obtained by *in vitro* synthesis, using STM images published in our previous paper (Radotić et al. 1998b). We compared this obtained fractal dimension with the fractal dimension of the enzymic polymer. The aim of this study was to obtain single fractal dimension of photochemical lignin, as well as to find out whether the fractal dimension of the polymer obtained with the applied method of lignin polymerization depends on a particular part of its surface, and to identify any correlation between the fractal dimension and the mechanism of lignin synthesis. In such a way, fractal analysis may help to compare the structural organization of lignin polymers obtained by different polymerization mechanisms.

Materials and Methods

The previously published STM images of photochemical lignin polymer (Radotić et al. 1998b) were used for determination of their fractal dimension.

The photochemical polymer (PCP) of coniferyl alcohol was synthesized by UV irradiation of a 2 × 10^{-2}M solution of coniferyl alcohol for two hours, using a 120 W low-pressure mercury lamp with a line emission spectrum. The intensity of UV radiation, determined by an oxalate actinometer, was 2 × 10^{12} quanta/s·cm^2 at the surface of the sample.

The resulting suspension of PCP was centrifuged at 3000 × *g*. The precipitate was resuspended in bidistilled water and recentrifuged. This procedure was repeated twice. Finally, the precipitate was air-dried.

For STM imaging, the precipitate was resuspended in bidistilled water. One drop of diluted suspension was deposited onto the substrate for STM images and evaporated in vacuum at 5°C. Gold plates were used as the substrate for STM imaging. The plates were made by vacuum deposition of gold in a custom-made device. The STM images were obtained using a commercially available model of the microscope (Nanoscope II, Digital Instruments). All STM images were recorded under ambient conditions using a Pt/Ir tip which was mechanically sharpened.

The aforementioned images were scanned by a Hewlett Packard Scan JetII cx scanner and prepared for analysis using Aldus Photo Shop Styler 2.0. The digitized images of the polymer were converted to a graphic form that is more suitable for manipulation, by using Marr-Hildreth convolution algorithm (Marr and Hildreth 1980; Zeineth and Kyriakidis 1986) from gray scale to binary silhouettes. The obtained silhouettes of polymers are shown in Figures 1–3. These silhouettes were used to obtain one-pixel-wide border of the image of polymer structure, which was used as the object of the further quantitative studies.

Digitized polymer shapes were analyzed by the box-counting (square-covering) method (Mandelbrot 1983; Smith et al. 1989; Cross et al. 1994). The registered binary border-image of each examined structure was expressed with an ensemble of 512 × 512 pixels, and transformed from the gray level of 1–256.

The binary border-images of the structure were analyzed by superimposing them on a succession of square grids, containing increasing numbers of squares (with decreasing edge length of each of them), in order to test invariability of the shape in different scale range. The number of grid squares (boxes) that a polymer image contacted (irrespective of how many pixels of the border it contained) were counted. The log number of squares encountered was plotted against the log of the box edge length, as given by

$$D_{\mathrm{B}} = \lim_{\varepsilon \to 0} \frac{\log N(\varepsilon)}{\log(1/\varepsilon)}$$

where D_{B} is the box-counting fractal dimension of the lignin polymer at the given magnification level, ε is the side length of one box within the grid, and $N(\varepsilon)$ is the smallest number of boxes of side length ε required to completely cover the border of the object being measured (Smith et al. 1989). In a log-log plot of the $(1/\varepsilon)$ (x-axis) versus $N(\varepsilon)$ (y-axis) the linear approximation of the first degree polynoma ($y = A + Bx$) was performed, where the slope (B) of the least square fitted line presents the box-counting fractal dimension (Figs. 1 3). To evaluate the quality of the fit of regression lines, the coefficient of determination (R) was used (the higher the values the better the fit). The range of data points which provided the value of the coefficient of determination $R = 0.99$ was assumed to be the range in which materials show self-similarity. All these processes were performed on a personal computer system (Intel, PC-80486), using Molecular Dynamics Image Quant (3.3) and MicroCal Origin (3.0).

The values of fractal dimension (D_{B}) were obtained by applying the described and tested method on a representative sample. The obtained fractal dimensions of lignin at different organization levels were statistically analyzed applying analysis of variance (ANOVA) for the level of reliability of 95% ($p < 0.05$), using MicroCal Origin program (version 3.0).

Results and Discussion

The scanning tunneling microscopic (STM) study of the enzymic polymer (EP) of coniferyl alcohol (Radotic et al. 1994) provided direct evidence for the modular organization of the enzymic polymer, as well as for the long-range order within the polymer structure. These images conform to the following mechanism. Globular modules consisting of ~20 monomers are formed first. These subsequently polymerize to form supermodules, which in turn aggregate to yield a solid polymer. The bonds between the modules in a supermodule are weaker than the bonds between monomers within the modules. However, they are of covalent type, since both modules and supermodules exist as distinct molecular species in solution and are quite stable. On the other hand, supermodules are interconnected by intermolecular forces within their aggregates. These aggregates exist only within solid lignin, and cannot be detected in solution as independent molecular species. The bonds between supermodules are split in solution (Radotić et al. 1994, 1998a). These results evidence that enzymic polymerization of phenolic alcohols is an ordered process rather than a random statistical process, even under *in vitro* conditions.

The STM images of PCP on a gold plate (Figs. 1–3) show similar structural motifs to those of EP, with repeating spherical regions. Such structural characteristics might be taken as evidence for the final structure of PCP being formed *via* aggregation of structural units formed by previous polymerization of monomers, as found in the case of EP. However, these images show that PCP has a lower degree of order than EP (Radotić et al. 1998b). These results are in agreement with molecular mass distribution curves of the two polymers (Radotić et al. 1998b). The difference between the molecular mass distributions of the two polymers may be a consequence of different mechanisms of their synthesis.

The mean fractal dimension of the enzymic lignin polymer is 1.929 ± 0.021. The results of fractal analysis of EP show that, at the 95% level ($p < 0.05$), there is no significant difference between the groups of analyzed images at different levels of structural organization (Radotić et al. 2000). This suggests that enzymic lignin produced *in vitro* has a fractal structural organization, in spite of the fact that lignin polymers have a highly cross-linked structure which is formed from several

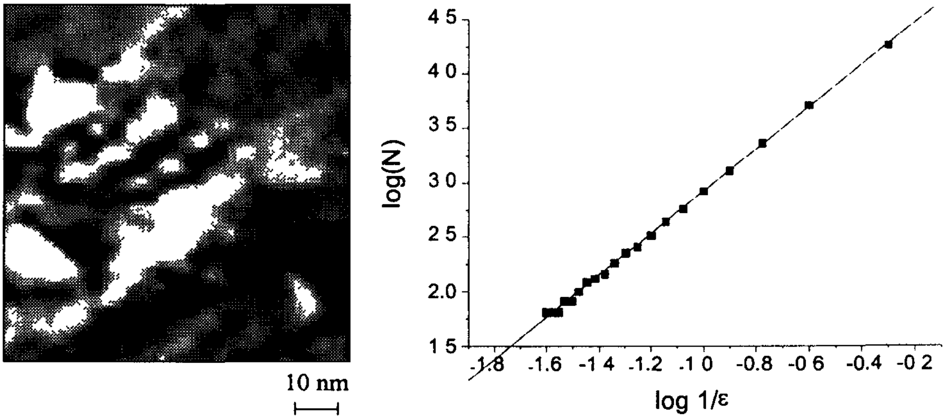

Figure 1. The binary silhouette of an analyzed fragment of STM image of the photochemical lignin polymer deposited onto gold substrate, and the corresponding log-log plot of $(1/\varepsilon)$ (x-axis) versus $N(\varepsilon)$ (y-axis) and linear approximation of the first degree polynoma ($y = A + Bx$), the slope (B) of the least square fitted straight line presents the box-counted fractal dimension (D_{B}) of the polymer, ε – the side length of the box, $N(\varepsilon)$ – the smallest number of boxes of side length ε required to completely cover the outline of the object been measured Data of the original STM image $U = -300$ mV, $I_{\mathrm{T}} = 1$ nA, $xy_{\mathrm{axes}} = 200$ nm

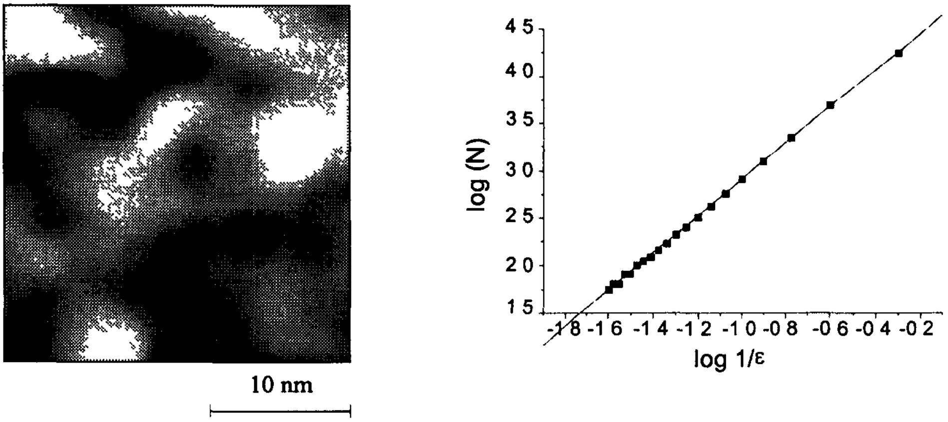

Figure 2. The binary silhouettes of an analyzed fragment of STM images presenting a lower organizational level of photochemical lignin polymer deposited onto gold substrate, and the corresponding log-log plots of ε versus $N(\varepsilon)$ Data of the original STM image $U = -300$ mV, $I_T = 1$ nA, $xy_{\text{axes}} = 60$ nm

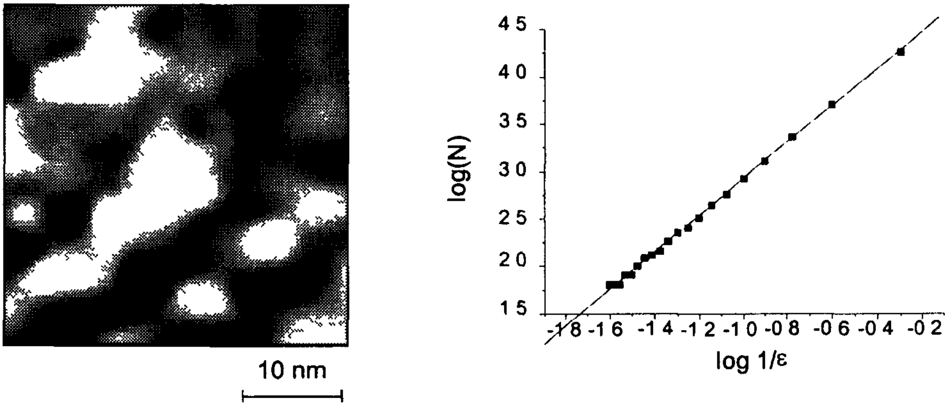

Figure 3. The binary silhouettes of an analyzed fragment of STM images presenting a lower organizational level of photochemical lignin polymer deposited onto gold substrate, and the corresponding log-log plots of ε versus $N(\varepsilon)$ Data of the original STM image $U = -300$ mV, $I_T = 1$ nA, $xy_{\text{axes}} = 80$ nm

multifunctional dimers and oligomers in the course of polymerization (Radotic et al 1997)

The mean fractal dimension of the photochemical lignin polymer at the higher organization level of the polymer, obtained using the image shown in Fig 1, is 1 938 ± 0 014 The mean fractal dimensions of the lower organization levels of the polymer, obtained using the images shown in Figs 2 and 3, are 1 941 ± 0 009 and

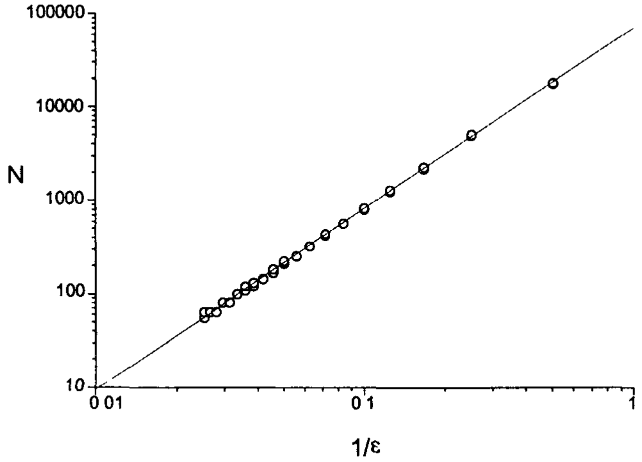

Figure 4. Log-log plot showing results of the box-counting method for all the analyzed images at different magnification scales on the same plot. The data follow a single line ($R = 0.999$).

1.937 ± 0.014, respectively. The mean fractal dimension of PCP is 1.939 ± 0.008. These data show that in the case of photochemical polymer, at the 95% level ($p < 0.05$), there is no significant difference between the groups of analyzed images at different levels of structural organization. That suggests the fractal structure of this polymer.

In order to provide an additional argument for the applicability of the fractal approach in our studies of lignin samples, we analyzed the obtained data in a still different way. We plotted the data of the box-counting method (i.e. fractal dimension) from the images at different magnification scales on the same diagram (Fig. 4). In addition to different scales of grid boxes necessary for the determination of fractal dimension, we also included one parameter more: different magnification scales of STM images of the polymer. In this way, log-log plot covers a much wider range of the box sizes and it is possible to have a single fractal dimension. The data follow single line ($R = 0.999$), showing that the fractal description can be applied to all studied sizes of the polymer. This provides an additional evidence that the fractal analysis of the polymer shapes is a reliable method which can be used for morphometrical purposes.

The fractal analysis of STM images shows that self-similarity characteristic features of both the enzymic and the photochemical lignin polymer remain the same on either enlargement or reduction of microscopic images, though it is evident from the images that PCP has a lower degree of ordering than EP (Figs. 1–3). The mechanism of coniferyl alcohol polymerization is different for the two polymers. It is known that the enzymic lignin polymer is formed through a free-radical mechanism of polymerization (Freudenberg et al. 1965). On the other hand, we have recently shown that photochemical lignin polymer is formed by an ionic mechanism (Radotić et al. 1997). The common feature of the mechanisms of formation of the two polymers is that in both cases dimers and oligomers are formed as intermediate products in the course of polymerization. Lignin polymers have a broad bimodal mass distribution (Wayman and Obiaga 1974; Radotić et al. 1994). However, very close values of fractal dimension for the two polymers obtained by different mechanisms of synthesis, as well as scaling dimensionality of the lignin images indicate that the building blocks (monomers, dimers, oligomers and modules) are cross-linked in a regular manner, even in *in vitro* conditions, independently of the polymerization mechanism. This fact proves that, though there are differences in the types of dimers and bonding patterns between the two polymers (Radotić et al. 1998a), and there is a lower degree of ordering in PCP (Radotić et al. 1998a,b), there is structural regularity at the higher organization levels (i.e. levels observable on the STM images) in both cases.

Based on the theoretical models, Ozol-Kalnin et al. (1986) calculated fractal dimensions of 1.78 ± 0.06 and 2.51 ± 0.06 for bulk and endwise polymerization, respectively. The two mechanisms differ in the rate of monomer introduction into the reaction volume. Endwise or chain-growth polymerization is characterized by successive additions of monomer phenoxy radicals to a few growing chains. The process is diffusion controlled since very small numbers of free radicals are present at any time. This mechanism has been proposed for living systems (Karmanov and Monakov 1995). Bulk polymerization, either polycondensation or polyaddition, however, is polymerization with random addition of all reactants (monomer, dimer and polymer molecules) to the polymer chains. In *in vitro* conditions, this mechanism of lignin synthesis is achieved by introduction of all monomers into solution in one step. In this case, a great number of polymerization centers are formed and react with each other.

Karmanov and Monakov (1995) studied fractal structure of lignin synthesized by *in vitro* enzymatic polymerization of ferulic acid. On the basis of hydrodynamic properties they found fractal dimensions of 1.66 ± 0.16 and 2.62 ± 0.3 for endwise and bulk polymerization, respectively. These results are quite opposite to those calculated by Ozol-Kalnin et al. (1986).

Fractal dimensions obtained for both enzymic and photochemical polymer, are close to that predicted for bulk polymerization (Ozol-Kalnin et al. 1986). Since we synthesized photochemical polymer by simultaneous irradiation of whole solution, it is expected that many reaction centers formed simultaneously and polymerization started independently from them by the polyaddition and/or polycondensation mechanism. This was further supported by mass distribution measurements and spectroscopic data (Radotić et al. 1994, 1997, 1998a). Spectroscopic data clearly show that both polymers are built from different fragments derived from transformed coniferyl alcohol monomers. The polymerization mechanism for the case of the enzymic polymer seems to be similar to that of photochemical lignin, and could be explained as cluster-cluster aggregation, comprising aggregation of structural units formed by polymerization of precursors This type of interaction corresponds to the bulk model of polymerization, which is in accordance with the results of fractal analysis.

PCP is an *in vitro* synthesized polymer There is no evidence that it is formed in natural conditions However, there is no reason to believe that such reaction might not be possible owing to the great photosensitivity of coniferyl alcohol and all of its polymeric products. The photochemical mechanism of lignin synthesis might have important ecological implications due to increasing UV radiation in the atmosphere as a consequence of stratospheric ozone depletion (Frederick et al 1989; Madronich 1992, Bojkov 1994). Increased UV radiation can be expected to result in an increased content of a more disordered photochemical lignin. Plants have probably developed some kind of enzymatic protective mechanism to destroy unwanted lignin structures However, if the threshold of the defense is passed, plants are probably not able to remove all irregular lignin structures, which might lead to the death of the plant cell Our expectations that fractal analysis made on the basis of STM images of the two polymers could differentiate between enzymic and photochemical lignin, have not fulfilled. The insignificant difference between fractal dimensions of the two polymers is not enough to be used as an unambiguous method.

Acknowledgements. This work was supported by the Ministry of Science of the Republic of Serbia

References

Bojkov R D (1994) The ozone layer – recent developments World Meteorol Org Bull **43**, 113—116

Cross S S, Cotton D W, Stephenson T J (1994) Progress in computer-generated three-dimensional reconstruction J Pathol **172**, 86—87

Falconer K (1990) Fractal Geometry Mathematical Foundations and Applications John Wiley, Chichester

Frederick J E, Snell H E, Haywood E K (1989) Solar ultraviolet radiation at the earth's surface Photochem Photobiol **50**, 443—450

Freudenberg K, Dietrich H (1953) Synthesis of D,L-pinoresinols and other investigations regarding lignin Chem Ber **86**, 1157—1166 (in German)

Freudenberg K, Chen C L, Harkin J M, Nimz H, Renner H (1965) Observations on lignin Chem Commun 224—225

Glenny R W, Robertson H T, Yamashiro S, Bassingthwaighte J B (1991) Applications of fractal analysis to physiology J Appl Physiol **70**, 2351—2367

Gluck J (1986) Chaos Making a New Science Penguin Books, New York

Karmanov A P, Monakov Yu B (1995) Fractal structure of *bulk* and *endwise* dehydropolymers Vysokomolekularnye soedinenia B **37**, 328—332 (in Russian)

Kokorevich A G , Gravitis Ya A , Ozol-Kalnin V G (1989) Development of the scaling approach in the investigations of lignin supramolecular structure Lignin as a fractal object Chim Drev No 1, 3—24 (in Russian)

Leary G (1971) Flash photolysis of coniferyl alcohol J Chem Soc D **13**, 688—689

Lewis N G , Inciong E J , Dhara K P , Yamamoto E (1989) High-performance liquid chromatographic separation of *E*- and *Z*- monolignols and their glucosides J Chromatogr **479**, 345—350

Lewis, N G , Yamamoto E (1990) Lignin occurrence, biogenesis and biodegradation Annu Rev Plant Physiol Plant Mol Biol **41**, 455—496

Madronich S (1992) Implications of recent total atmospheric ozone measurements for biologically active ultraviolet radiation reaching the earth's surface Geophys Res Lett **19**, 37—40

Mandelbrot B B (1983) The Fractal Geometry of Nature Freeman, New York

Marr D , Hildreth E (1980) Theory of edge detection Proc R Soc London , Ser B **207**, 187—217

Ozol-Kalnin V G , Kokorevich A G , Gravitis J (1986) Estimation of fractal and chemical dimensions of bulk and endwise polymers Chim Drev No 5, 108—109 (in Russian)

Rabouille C , Cortassa S , Aon M A (1992) Fractal organization in biological macromolecular lattices J Biomol Struct Dyn **9**, 1013—1024

Radotić K , Simić-Krstić J , Jeremić M , Trifunović M (1994) A study of lignin formation at the molecular level by scanning tunneling microscopy Biophys J **66**, 1763—1767

Radotić K , Zakrzewska J , Sladić D , Jeremić M (1997) Study of photochemical reactions of coniferyl alcohol I Mechanism and intermediate products of UV radiation – induced polymerization of coniferyl alcohol Photochem Photobiol **65**, 284—291

Radotić K , Todorović S , Zakrzewska J , Jeremić M (1998a) Study of photochemical reactions of coniferyl alcohol II Comparative structural study of photochemical and enzymatic polymer of coniferyl alcohol Photochem Photobiol **68**, 703—709

Radotić K , Budinski-Simendić J , Jeremić M (1998b) A comparative structural study of enzymic and photochemical lignin by scanning tunneling microscopy and molecular mass distribution Chem Ind **52**, 347—350

Radotić K , Tasić M , Jeremić M , Budimlija Z , Simić-Krstić J , Polzović A , Božović Z (2000) Fractal analysis of STM images of lignin polymer obtained by *in vitro* synthesis Gen Physiol Biophys **19**, 171—180

Ramakrishnan A , Sadana A (1999) Analysis of analyte-receptor binding kinetics for biosensor applications an overview of the influence of the fractal dimension on the surface on the binding rate coefficient Biotechnol Appl Biochem **29**, 45—57

Shumilin V A , Zhurbilo L A (1988) One-dimensional modeling of the initial stage of dehydrogenative polymer synthesis, as a diffusion controlled process Chim Drev No 3, 59—66 (in Russian)

Smith J T G , Marks W B , Lange G D , Sheriff W H , Neale E A (1989) A fractal analysis of cell images J Neurosci Methods **27**, 173—180

Wayman M , Obiaga T I (1974) The modular structure of lignin Can J Chem **52**, 2102—2110

Yamane S , Takayama K , Nagai T (1998) Effect of fractal dimension on drug permeation through porous ethylcellulose films J Controlled Release **50**, 103—109

Zeineth R A , Kyriakidis G (1986) Use of reflectance in soft laser densitometry in scanning opaque electrophoregrams A high-resolution technique Appl Biochem Biotechnol **12**, 25—30

Final version accepted December 2, 2000