Fractal Analysis of STM Images of Lignin Polymer Obtained by *in vitro* Synthesis

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, 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*
4 *Laboratory for Molecular Machines, Mechanical Faculty, University of Belgrade*
5 *Department of Anatomy, Faculty of Medicine, University of Novi Sad, Novi Sad, Yugoslavia*

Abstract. Lignin, the structural polymer of the plant cell walls, is produced by free radical polymerization of phenolic alcohols, catalyzed by different peroxidases. The mechanism and the structural organization of lignin in the cell have not been completely understood. In this study we applied fractal analysis to images of lignin polymer obtained using scanning tunneling microscope. The analysis showed the regularity of the polymer at different levels of organization. According to the results obtained, at the 95% confidence level, there is no significant difference in the fractal dimension between images representing different organizational levels of lignin. In other words, lignin produced in *in vitro* conditions has fractal structural organization and, consequently the polymer can be expected to be regular in *in vivo* conditions. The value of the fractal dimension 1.929 ± 0.021 is in good agreement with the theoretically predicted value for polyaddition and polycondensation mechanism of polymerization. The mechanism of *in vivo* lignin synthesis is discussed in terms of various experimental and theoretical evidences. In this paper, we could show that fractal analysis of the lignin polymer is a useful complementary approach to the experimental data collection in structural and phenomenological studies.

Introduction

The possibility of analysing and quantifying the attributes of natural shapes at both the micro and the macro level has important implications in the structural

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

studies in many fields. Visualization of microscopically observable shapes and their analysis are improved and facilitated by using computer technology and applicable program solutions.

The concept of "fractal geometry", introduced in science by Mandelbrot (1983) has been used in various scientific disciplines during several recent years. However, the transition between aesthetic contemplation of fractal images and application of fractal geometry to the analysis of naturally occuring images has been relatively slow (Cross et al. 1994a). The principal concept of fractal geometry is based on the fact that most shapes in the nature cannot be described well enough 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 amount of detail as the image is viewed at different levels of magnification, i.e. different range of scale (Gluck 1986; Falconer 1990) Based on these facts methods were developed for "complexity measurements" of different natural shapes (Glenny et al. 1991; Cross et al. 1994a; Cross et al. 1994b; Khokha 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 could be demonstrated between fractal organization and biological activity of macromolecules, with possible practical applications of this approach. Thus, Ramakrishnan and Sadana (1999) have used single- and dual - fractal analysis to study analyte-receptor binding kinetics for different types of biosensor applications. The predictive relationships they obtained provide further physical insights into the binding reactions on the surface, and should assist in enhancing biosensor applications. Yamane et al. (1998) applied fractal analysis to quantify the complexity of a porous film prepared with ethylcelulose and diethylphtalate as plasticizer. The effect of fractal dimension on the drug permeation through the films was evaluated, showing the possibility to use this approach in designing drug release systems. Such results may be of importance in studying possible relationships between geometry of macromolecular organization and some biological functions.

Lignin, the structural polymer of the plant cell walls and the second most abundant natural polymer on the Earth, is formed through free radical polymerization of phenolic alcohols (coniferyl, *p*-coumaryl and synapyl alcohol) catalyzed by different peroxidases. It is widely accepted that lignin has a protective role in the plant cell against different kinds of stress (Lewis and Yamamoto 1990). However, much remains unknown about the mechanism of synthesis and structural organization of lignin, and especially about the regularity of the polymer structure in the cell wall.

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 this study, lignin fractal structure is investigated for the first time on the

basis of directly observed microscopic images of its structure. The aim of this paper is to report on the quantitative evidence that the process of lignin polymerization in *in vitro* conditions is highly ordered; to obtain the single fractal dimension of lignin in the studied process at different levels of magnification; to find out whether the fractal dimension of the polymer with the respect to the given method of lignin polymerization depends on a particular part of its surface; and to find out if there is any correlation between fractal dimension and mechanism of synthesis.

Materials and Methods

The previously published STM images of lignin polymer synthesized *in vitro* (Radotić et al. 1994) were used for determination of its fractal dimension.

Enzymatic lignin for microscopic samples was synthesized from 5×10^{-3} mol/l coniferyl alcohol and 5×10^{-3} mol/l H_2O_2 in presence of 2.5×10^{-8} mol/l horseradish peroxidase, in 5×10^{-2} mol/l phosphate buffer pH 7.6. The polymer structure was studied two days after the start of polymerization. The STM images of the polymer were obtained using a commercially available model of the microscope (Nanoscope II, Digital Instruments). The images were recorded using a Pt/Ir tip which was mechanically sharpened. Highly oriented pyrolytic graphite (HOPG) plates were used as the substrate for polymer samples (Radotić et al. 1994).

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) from gray scale to binary silhouettes. The obtained silhouettes of polymers are shown in the Figures 1–3. These silhouettes were used to obtain one-pixel-wide border of the image of the 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; Peitgen et al. 1992; Cross et al. 1994a). The registered binary border-image of each examined structure was expressed as 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 number of squares (with decreasing edge lengths of each of them), in order to test invariability of the shape in a different scale range. The number of grid squares (boxes) the polymer image contacted (no matter how many pixels of the border it contained) were counted. The log number of squares counted 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

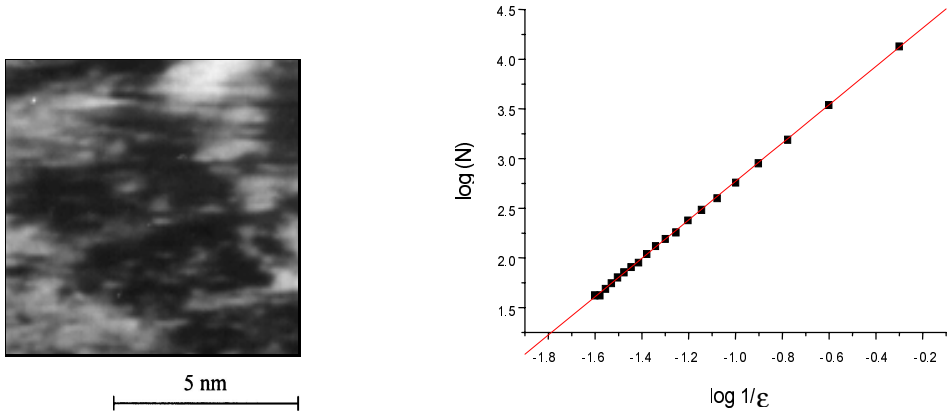

Figure 1. The binary silhouette of the analyzed fragment of STM image of the enzymatic lignin polymer deposited onto HOPG substrate, and the corresponding log-log plot of the $(1/\varepsilon)$ (x-axis) versus N(ε) (y-axis) and linear approximation of the first degree polynomial $(y = A + Bx)$; the slope (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 measured. Data for the original STM image (Radotić et al. 1994): $U = 100.1$ mV, $I_{\mathrm{T}} = 0.80$ nA, $xy_{\mathrm{axes}} = 15$ nm.

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 polynomial $(y = A + Bx)$ 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 the regression lines, the coefficient of determination (R) was used (the higher its value 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 the present materials show self-similarity. All these processes were run 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 95% level of reliability $(p < 0.05)$, using MicroCal Origin program (version 3.0).

Results and Discussion

Fractal analysis of macromolecular species that build the cell walls is a new useful approach to further our knowledge about the architecture of this cell compartment.

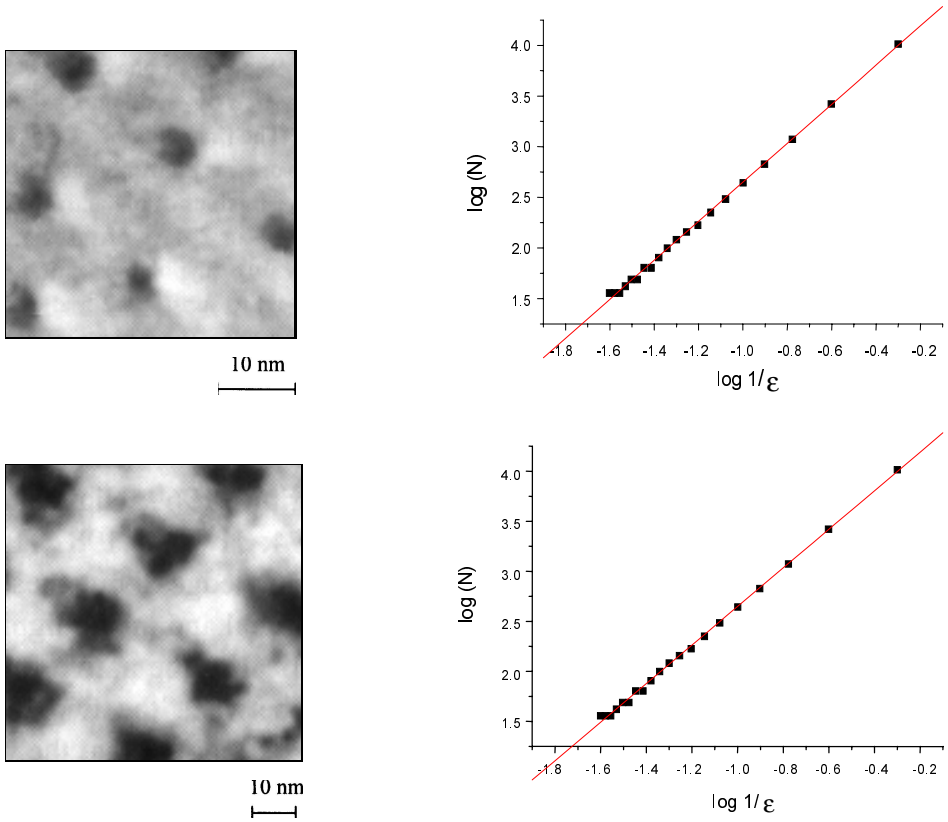

Figure 2. The binary silhouettes of the analyzed fragments of STM images presenting two different structural motifs of the enzymatic lignin polymer deposited onto HOPG substrate, and the corresponding log-log plots of ε versus $N(\varepsilon)$. Data for the original STM image: a) $U = 20.1$ mV, $I_{\mathrm{T}} = 0.50$ mA, $xy_{\mathrm{axes}} = 80$ nm; b) $U = 180.1$ mV, $I_{\mathrm{T}} = 0.46$ nm, $xy_{\mathrm{axes}} = 100$ nm.

Fractal analysis was recently performed in studies of certain polysaccharides, the molecular species that are one of the building units of the cell walls. An example is the use of surface fractal analysis approach to study morphological differences between particles obtained by association between indomethacin and β -cyclodextrin, made by exposing their physical mixture to the ultrasound (Fini et al. 1997). Lignin differs from the other constituents of the cell walls in that it is built from phenolic monomers.

Figures 1–3 present binary silhouettes of the original STM images obtained at descending magnification levels of the polymer samples, together with the corresponding log-log plots which were used for the determination of the fractal dimension. The mean fractal dimension of the lowest organization level of the lignin

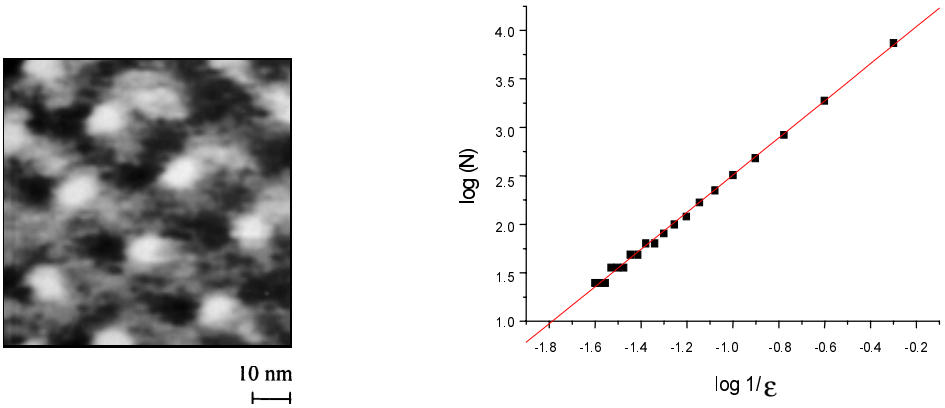

Figure 3. The binary silhouettes of the analyzed fragment of STM image of the enzymatic lignin polymer deposited onto HOPG substrate, and the corresponding log-log plot of ε versus $N(\varepsilon)$. Data for the original STM image: $U = 20.1$ mV, $I_{\mathrm{T}} = 0.50$ nA, $xy_{\mathrm{axes}} = 100$ nm.

polymer obtained using the image shown in the Fig. 1, was $D_{\mathrm{B}} = 1.932 \pm 0.008$ ($R = 0.9999$). The mean fractal dimensions of two different structural motifs of the polymer at the higher organization level obtained using images shown in Radotić et. al. 1994. (Fig. 2) were $D_{\mathrm{B}} = 1.926 \pm 0.016$ ($R = 0.9993$) and $D_{\mathrm{B}} = 1.927 \pm 0.016$ ($R = 0.9993$). The fractal dimension of the highest observed organization level (the lowest microscope magnification) of the polymer (Fig. 3) was $D_{\mathrm{B}} = 1.916 \pm 0.020$ ($R = 0.9990$). The mean fractal dimension of the lignin polymer is $D_{\mathrm{B}} = 1.929 \pm 0.021$ ($R = 0.0991$). The results show that, at the 95% ($p < 0.05$) level there is no significant difference between the groups of analyzed images at different levels of structural organization. This suggests that lignin produced *in vitro* has a fractal structural organization, in spite of the fact that lignin polymers have a highly cross-linked structure formed from several multifunctional dimers and oligomers in the course of the polymerization (Radotić et al. 1998). The regularity of lignin has been demonstrated for the first time by visual observation of STM images (Radotić et al. 1994) which show an apparent crystal-like organization within the polymer. Fractal analysis of STM images shows that lignin self-similarity characteristic features remain the same on either enlargement or reducing of microscopic images.

In order to provide an additional argument for the applicability of the fractal approach in studies of our lignin samples, we analyzed the obtained data from a different aspect. Data obtained from the box-counting method (i.e. fractal dimension) from images at different magnification scales were put on the same plot (Fig. 4). Beside the fact that different scales of grid boxes are necessary for the determination of the fractal dimension, an additional parameter is included in such an analysis: different magnification scales of STM images of the polymer. In this

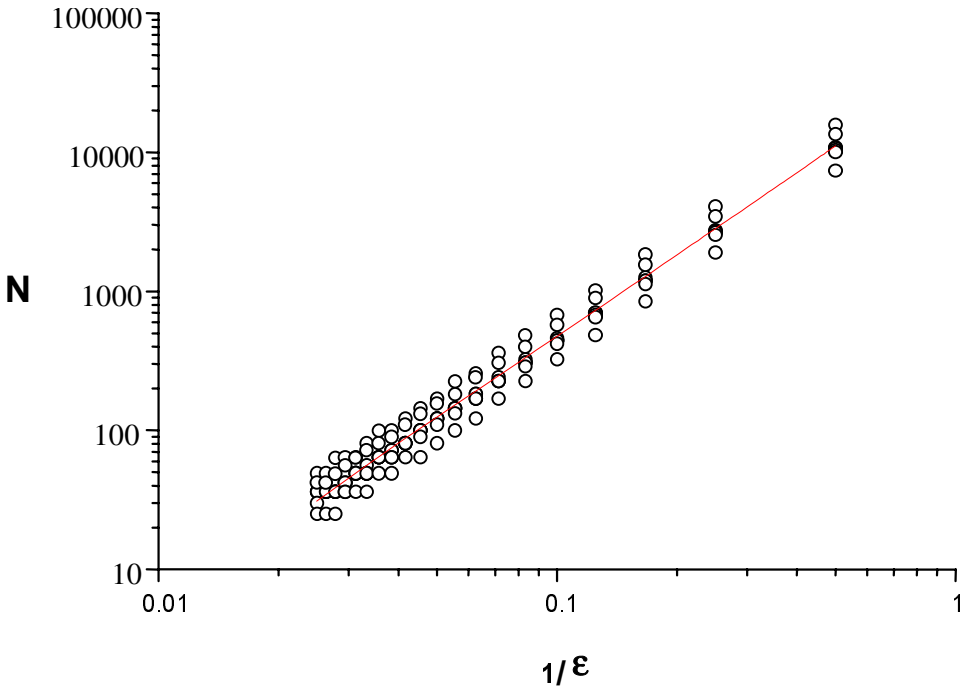

Figure 4. Log-log plot showing the results of the box-counting method from all the analyzed images at different magnification scales on the same plot. The data are grouped along a single line ($R = 0.975$).

way, the log-log plot covers a much wider range of box sizes and a single fractal dimension can be obtained. The data are distributed along a single line ($R = 0.975$) showing that the fractal description can be applied to all studied sizes of the polymer. This is an additional evidence that fractal analysis of the polymer shapes is a reliable method which can be used for morphometrical purposes.

In order to show the accuracy of the modified system applied for the evaluation of the fractal dimension, fractal analysis of some typical geometrical figures and mathematically described fractal patterns was performed using the same analytical procedure. The values of the determined fractal dimension were compared with the theoretically expected values. The fractal dimensions determined and the observed errors are presented in Table 1. It is worth noting that the lignin fractal dimension $D_{\mathrm{B}} = 1.929$ is close to $D_{\mathrm{B}} = 2$, a characteristic for the surface fractal dimension of an Euclidian body. The lignin polymer is not composed of Euclidian bodies, but apparently polymer chains are arranged as regular objects giving them a crystal-like feature.

Lignin polymers have a broad bimodal mass distribution (Wayman and Obiaga 1974; Radotić et al. 1994). However, the scaling dimensionality of the lignin

Table 1. Calculated fractal dimensions of some geometrical figures versus fractal dimensions determined for the lignin polymer in this paper; D_B – fractal dimension; R – coefficient of determination

Subject	Expected D_B	D_B	R	Error (%)
Line	1.00	0.9893 ± 0.023	0.998	1.07
Circle	1.00	1.0405 ± 0.11467	0.998	4.05
Koch snowflake	1.2618	1.22183 ± 0.02475	0.999	3.17
Sierpinski carpet	1.8928	1.81823 ± 0.07034	0.998	3.94

images suggests that a multifunctionality of the building blocks (monomers, dimers, oligomers and modules) are cross-linked in a regular manner, probably due to the enzyme action, even in *in vitro* conditions.

Based on the theoretical models, Ozols-Kalnin et al. (1986) calculated fractal dimension values 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 in the reaction volume. Endwise or chain-growth polymerization is characterized by successive addition of monomer phenoxy radicals to a few growing chains. The process is diffusion controlled since very small number of free radicals is 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 introducing 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 the fractal structure of lignin synthesized by *in vitro* enzymatic polymerization of ferulic acid. On the basis of hydrodynamic properties they found fractal dimension values of 1.66 ± 0.16 and 2.62 ± 0.3 for endwise and bulk polymerization, respectively. These results are quite different from those calculated by Ozols-Kalnin et al. (1986). On the other hand, fractal dimension values obtained on the basis of hydrodynamic data for isolated lignins varied between 1.79 and 2.68 depending on the extent of polymer destruction during the isolation procedure and the type of solvent used (Kokorevich et al. 1989). This shows that none of the fractal dimensions obtained can be used to represent the native lignin polymer *in vivo*.

The value of the fractal dimension $D_B = 1.929$ obtained in this study is close to the predicted one for bulk polymerization (Ozols-Kalnin et al. 1986). Since we synthesized lignin polymers by simultaneous mixing of all reactants, it may be expected to have reacted by the polyaddition and/or polycondensation mechanism. Further support to this assumption is provided by mass distribution measurements and spectroscopic data (Radotić et al. 1994, 1997, 1998). Spectroscopic data clearly

show that the polymers were built from different fragments derived from transformed coniferyl alcohol monomers. Although STM images give the surface pattern of the polymer, it seems from this consideration that it bears the mechanism of synthesis in itself.

In this study, the results were obtained from *in vitro* prepared polymers. However, the results suggest that the endwise and the chain-growth mechanism (proposed for living system) should be reconsidered. There are low amounts of coniferyl radical and coniferyl alcohol naturally present in the cell wall (Lewis and Yamamoto 1990), approximately equal to the concentrations used for our *in vitro* synthesis. The two components are not added immediately to the polymerization center. Both species are multifunctional monomers which readily form various dimers and oligomers. That property can easily give rise to the other, i.e. bulk mechanism.

Fractal analysis of the lignin polymer may be a very useful complementary approach to experimental data collection with respect to both main aspects of polymer studies, structural and phenomenological. The fractal analysis of lignin formed by different mechanisms in *in vitro* conditions and its comparison with fractal analysis of natural lignin could contribute to the understanding if the fractal dimension obtained in this paper is a genuine feature of lignin formation *in vivo*. On the other hand, it would be useful to find a suitable method as the basis for determination of fractal dimension of lignin *in vivo,* as well as to find out whether it is possible to use this fractal dimension for non-invasive qualitative and quantitative evaluation of lignin production and changes of its structure with changing environmental conditions. This would provide a further insight into the role of lignin in plant cell wall functioning and its reactions to external stress.

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

References

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

Cross S. S., Howat A. J., Stephenson T. J., Cotton D. W., Underwood J. C. (1994b): Fractal geometric analysis of material from molar and non-molar pregnancies. J. Pathol. **173**, 115—118

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

Fini A., Fernandez-Hervas M. J., Holgado M. A., Rodriguez L., Cavallari C., Passerini N., Caputo O. (1997): Fractal analysis of β-cyclodextrin-indomethacin particles compacted by ultrasound. J. Pharm. Sci. **86**, 1303—1309

Glenny W. R., 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. Visokomolekularnie soedineniya **B37**, 328—332 (in Russian)

Khokha M. K., Landini G., Iannaccone P. M. (1994): Fractal geometry in rat chimeras demonstrates that a repetitive cell division program may generate liver parenchyma. Develop. Biol. 165, 545—555

Kokorevich A. G., Gravitis Ya. A, Ozol-Kalnin (1989): Development of scaling approach in studies of supramolecular structure of lignin. Lignin as a fractal object. Chim. Drev. **1**, 3—24 (in Russian)

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

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

Marr D., Hildreth E. (1980): Theory of edge detection. Proc. Roy. Soc. London **207**, 187—217

Ozols-Kalnin V. G., Kokorevich A. G., Gravitis J. (1986): Estimation of fractal and chemical dimensions of bulk and end-wise polymers. Chim. Drev. **5**, 108—109 (in Russian)

Peitgen H. O., Jurgens H., Saupe D. (1992): Fractals in the Classroom: Part One – Introduction to Fractals and Chaos. Springer Verlag, New York

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. (1998): Study of photochemical reactions of coniferyl alcohol. II. Comparative structural study of a photochemical and enzymatic polymer of coniferyl alcohol. Photochem. Photobiol. **68**, 703—709

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. **3**, 59—66 (in Russian)

Smith T. G. Jr., Marks W. B., Lange G. D., Sheriff W. H. Jr., Neale E. A. (1989): A fractal analysis of cell images. J. Neurosci. Meth. **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. Controll. Rel. **50**, 103—109

Final version accepted March 6, 2000