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Evolution of erythrocytes aggregation: A fractal approach when incubated with *Trichinella spiralis* and *Ascaris lumbricoides*

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ABSTRACT

Fractal geometry has provided a new range of concepts that could explain the non-linear behaviour of seemingly unpredictable natural systems. The aim of this work is to apply nonlinear analysis to study the *in vitro* erythrocyte aggregation produced by incubation of human red blood cells (RBCs) with *Ascaris lumbricoides* and also *Trichinella spirallis* larvae extracts. The approach used was based on calculating the fractal slope (D), through image processing of control and treated RBCs at different incubation times (200 images). The results showed a decrease in the fractal dimension value of *Ascaris lumbricoides* treated RBCs (up to 10%) and a constant value of D in the case of *Thichinella spiralis* treated RBCs, in contrast to a more disperse value showed in normal controls. This modification in the D might be correlated with the different actions of each parasite over the erythrocyte membrane leading to abnormal RBCs aggregation.

1. Introduction

Trichinella spiralis is a pathogen parasite involved in the development of trichinosis. Its infectious cyst form is the whipworm. During muscular larvae encystment, an intense immune response takes place, which can be avoided by the parasite to ensure its presence in the host. Additionally, *Ascaris lumbricoides* parasites produce ascariasis, one of the most widespread parasitic diseases in the world. Its most characteristic effects are perforation of the intestinal wall and pulmonary abscesses.

Red blood cells (RBCs) are the most abundant cells in the blood and its primary function is transport of oxygen and carbon dioxide. Due to its mechanical properties and abundance, RBCs are also major determinants for the rheological behaviour of blood. It has been shown that lower amount of surface sialic acid not only increases cell aggregation but also blood viscosity and flow disorder. Possible changes in the erythrocytes membrane, by *in vitro* interaction with new borne larvae of the parasites, were evaluated analysing the alterations in erythrocyte aggregation by image analysis [1]. Cellular mechanisms of many infectious processes are not exactly known, but certain compounds found in the *Ascaris lumbricoides* parasite surface are proposed to cause a significant decrease in the superficial sialic acid of RBCs, resulting in an increase in erythrocyte aggregation [2]. This phenomenon may be related to some aspects of the pathology, such as thrombus formation and anaemia. Cellular mechanisms involved in this process are not clearly known. Previous publications have reported that host and parasite glyco-conjugates are involved in this response. Moreover, our research group has demonstrated the capture of erythrocyte sialic acid by muscle larvae, suggesting the possibility of interaction between the parasite and muscle cells [3].

For a quantitative description of both phenomena various techniques have been described [4–7] without conclusive results, as the examined biological system has complex features. In this work we develop a feasible alternative, based on fractal patterns measured at different portions of the samples. This method applies a parameter that allows the discrimination of different groups according to their nature. In general, we could define a fractal as a geometric figure with very complex structure at any scale that is impossible to define by the concepts and methods developed since Euclides. Therefore, the measurement of fractal shapes involves new concepts beyond the classical geometrical ones. Fractal dimensions cannot be calculated accurately but might be

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estimated. There are different definitions for fractal dimension [8]. Simple methods for estimating fractal dimension are correlation dimensions [9] and information dimension, which require specific assumptions and are restricted to time series analysis [10]. The most celebrated one is Hausdorff dimension [11], while the most popular one is the box-counting dimension. Although in many cases Hausdorff dimension equals the box-counting dimension, in general the Hausdorff dimension is used only in theoretical settings and is too subtle for practitioners [12]. A widespread strategy to estimate the fractal dimension is box counting, which is the method used in our work. Box counting method is undoubtedly the most widespread in the scientific literature. It can be applied to any distribution points, curves, surfaces, etc., and is the one chosen in our work, especially because we are dealing with pictures, not with time series.

This article details how the fractal slope was used to determine possible changes due to *in vitro* contact of RBCs with *Ascaris lumbricoides* and *Trichinella spiralis* muscle larvae extracts, using image processing free software FRACTALYSE [13].

2. Materials and methods

2.1. Parasites and red blood cells (RBCs)

2.1.1. Parasite extracts (PE)

Firstly, five PE of adult *Ascaris lumbricoides* at concentrations of 5100 ± 200 larvae/mL were obtained by surgical removal of the cuticle and refrigerated mechanical rupture, following the protocol described in Ref. [3].

Secondly, infective larvae of *Trichinella spiralis* obtained from muscle of infected mice were released by artificial digestion using pepsin and hydrochloric acid. The larvae were concentrated by centrifugation and counted by duplicate. Five concentrates of PE were prepared with an amount of muscle larvae of 4300 ± 200 larvae/mL.

2.1.2. Red blood cell suspensions

All human beings involved in this work sign a medical informed consent form for participating in this research work. The ethics committee of the Rosario National University approved this project (Resolution number: 033/2015).

Fresh group O blood samples were collected from a healthy donor into a container holding EDTA as anticoagulant, stored at 4 °C and analysed within 24 h. After removing autologous plasma, RBCs were washed three times with phosphate-buffered saline (PBS) (pH = 7.4, 295 ± 8 mOsmol/kg).

2.1.3. Treatment of RBCs

The erythrocyte pellet was incubated with equal volume of PE for 0, 60 and 90 min at 37 °C (treated RBCs). The RBCs used as control (control RBCs), were incubated only with PBS. After the incubation, the treated and control RBCs were washed with PBS.

2.2. Data analysis

In this work, we provide an easy-going explanation to the box counting dimension concept. To calculate this dimension, imagine the fractal lying on an evenly spaced grid, and count how many boxes are required to cover the set. The Box counting dimension is calculated by seeing how this number changes as we make the grid finer by applying a Box-counting algorithm. Suppose that $N(L)$ represents the number of boxes of side L required to cover the set. Then the box counting dimension is defined as:

$$Dim(X) = \lim_{L\to 0} \frac{logN(L)}{log\left(\frac{1}{L}\right)} \tag{1}$$

Rigorously defined Box counting dimension of the set $X \subset \Re^n$, $n = 2$, is defined as follows:

The lower Box counting dimension:

$$\underline{Dim}(X) = \lim_{L\to 0}\ inf\ \frac{logN(L)}{log\left(\frac{1}{L}\right)} \tag{2}$$

Similarly the upper Box counting dimension:

$$\overline{Dim}(X) = \lim_{L\to 0}\ sup\ \frac{logN(L)}{log\left(\frac{1}{L}\right)} \tag{3}$$

If $\overline{Dim}(X) = \underline{Dim}(X)$, then the common value is called the box counting dimension. Thus, the box counting dimension is defined by:

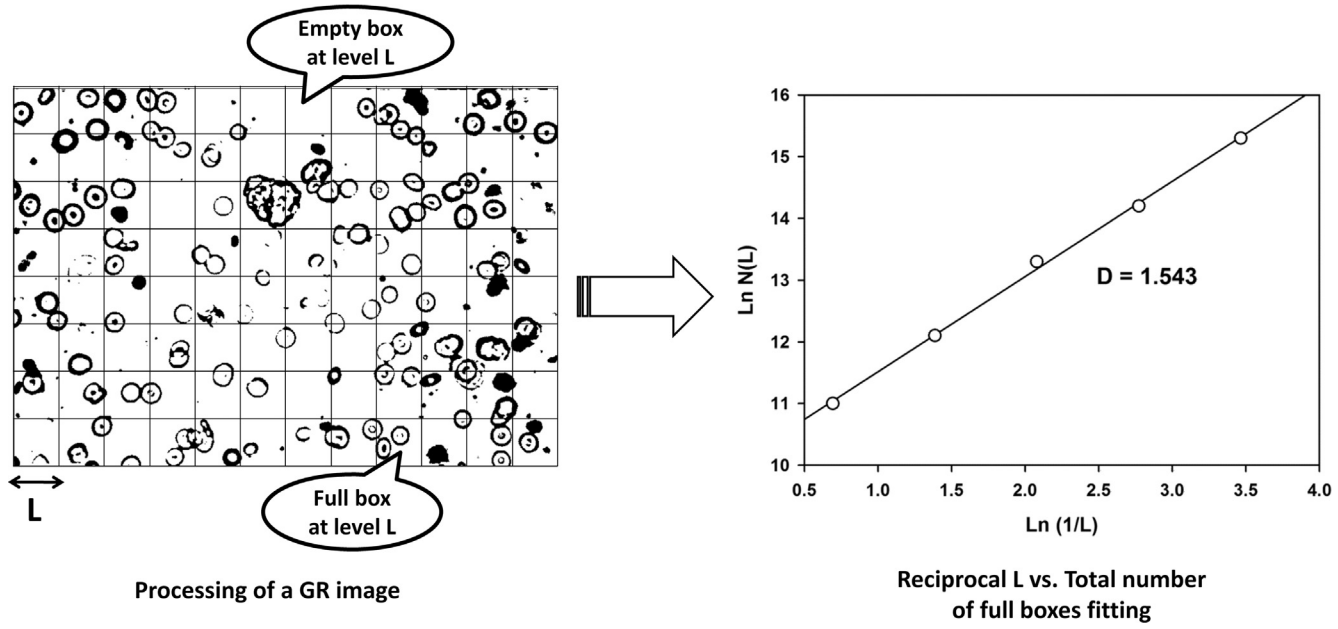

Figure 1. Box counting method, using an example: D is the value of the slope of the line resulting from the level ratio of each graph (1/L) versus the number of occupied spaces in that level (N (L)).

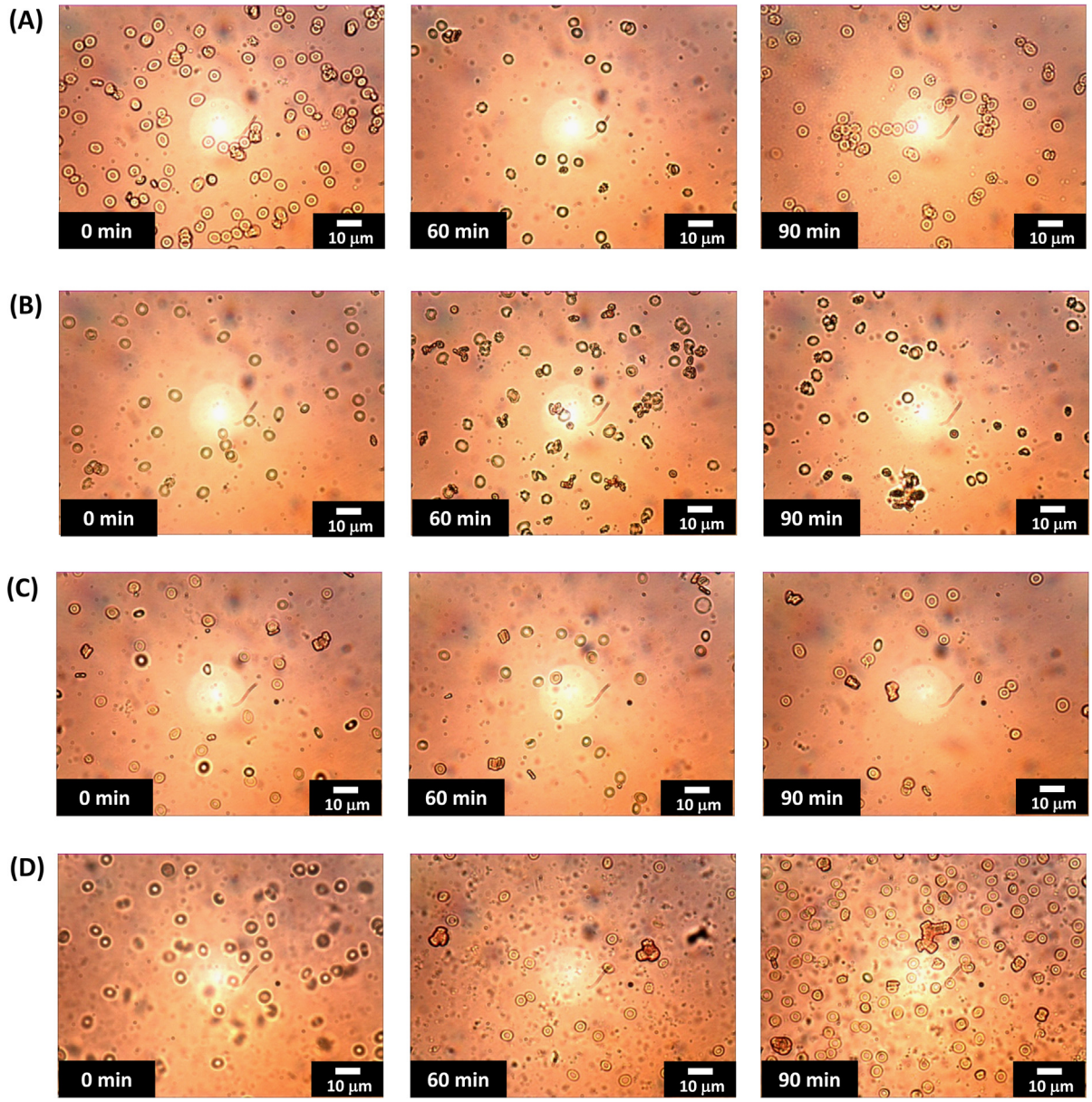

Figure 2. Images of control RBCs (A), Ascaris treated RBCs (B), control RBCs (C) and Trichina treated RBCs (D) taken at different times of test, all at 40× magnification. The white line as a scale bar represents 10 µm in each figure.

$$Dim(X) = \lim_{L \to 0} \frac{logN(L)}{log\left(\frac{1}{L}\right)} \tag{4}$$

if such limit exist.

As working with experimental time series the process itself defined the minimum number of boxes of side L required to cover the set, then:

$$Dim(X) = \frac{logN(L)}{log\left(\frac{1}{L}\right)} \tag{5}$$

2.3. Digital image analysis

Treated and control RBCs were suspended in 0.13% autologous plasma and maintained at rest for 5 min to allow aggregation. Five RBCs control samples containing equal amount of parasite extracts were examined in a concave optical inverted microscope slide (Union Optical, Japan). Images for each sample (60 images analysed) were obtained by duplicate (objective: 40X, Canon Powershot A640 digital camera). Images were then processed with the software FRACTALYSE. Fractal dimension (D) was calculated according to an iterative principle based on the box counting method.

Fig. 1 shows an example of the analysis strategy. On each fractal image (automatically transformed into black and white, to avoid impurities) FRACTALYZE overlays a grid of squares or boxes of side L. Then, filled boxes are counted (N) and procedure is repeated for different values of L, in this work we used L = 16. Finally, results are showed on a graph where abscissas represent the absolute values of the logarithms of reciprocal L, (Ln 1/L), and ordinates correspond to logarithm of N. Therefore, the slope of the resulting line is the fractal dimension according to Eq. (5). If the obtained relationship does not fit a power law, the object is not self-similar or fractal.

The FRACTALYSE software was used as a tool for automatizing D calculation by the box counting method in images obtained from treated and controls RBCs, to develop a technique that might be useful for detection of RBCs membrane modifications.

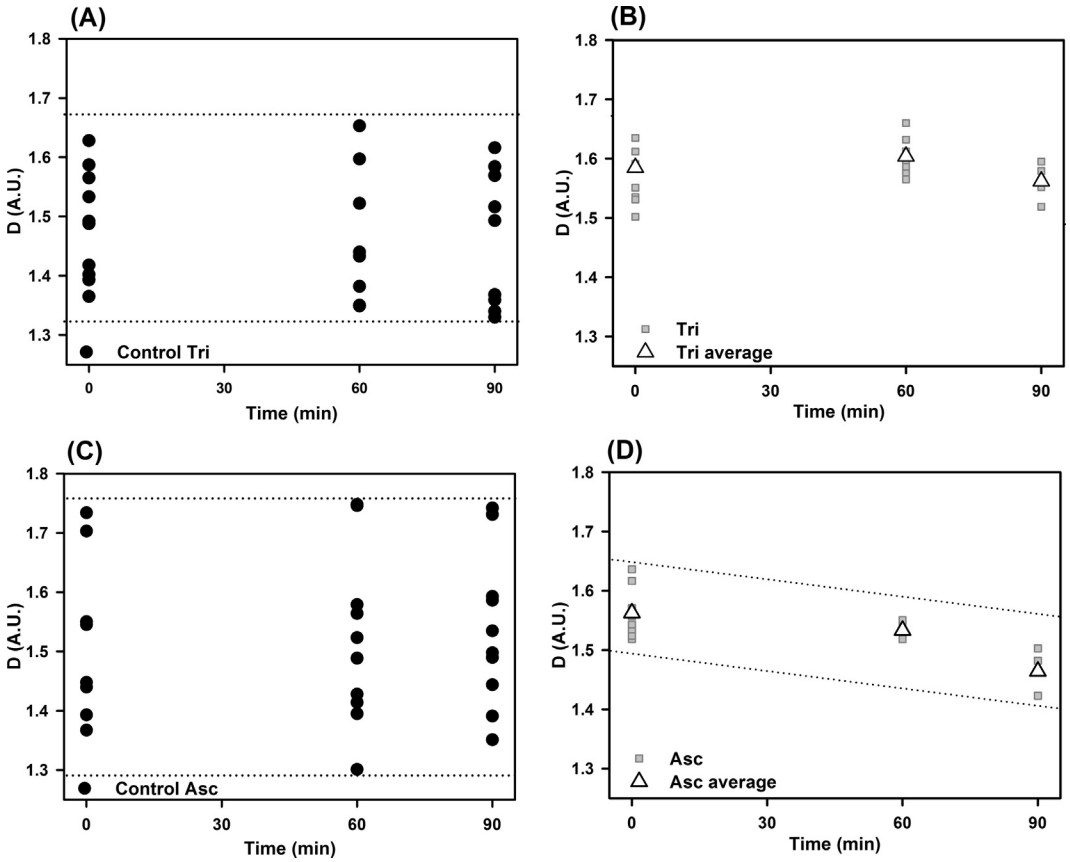

Figure 3. Comparison between the corresponding fractal slopes of all the samples analysed for both parasites: control RBCs (A), Trichina treated RBCs (B), control RBCs (C) and Ascaris treated RBCs (D) taken at different times of test. The dotted lines are placed to aid the result interpretation.

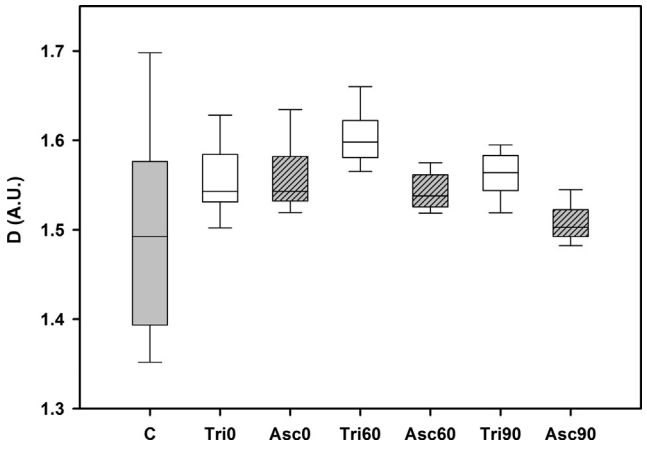

Figure 4. Box plots corresponding to dispersion of fractal slopes over the samples analysed for both parasites.

3. Results and discussion

Fig. 2 shows representative images of control and treated RBCs taken at different times. Differences in the distribution of red cell aggregates between the groups are evident. Moreover, increase of incubation time also generates visible differences in the treated RBCs images. These are consistent with semi-quantitative results reported previously, where it was shown that treated RBCs showed an increase in aggregate with a marked decrease in the number of the isolated cells [3].

Comparison between the corresponding fractal slopes of all the samples analysed for both parasites, and comparison with their respective controls is shown in Fig. 3. Control RBCs are never in contact with the parasite while RBC incubated with the parasite extract for 0 min are images obtained immediately after the parasite extract was added to the sample. It can be seen that in both cases the control samples have a highly disperse random variation of fractal slopes, while in the case of treated RBCs exhibit minor dispersions in each case. For *Ascaris lumbricoides* treatment, a decreasing behaviour is observed on average fractal slopes compared with the randomness observed in controls at the same times. Regarding *Trichinella spiralis,* the fractal slopes remains nearly constant over a certain value, nevertheless presenting greater randomness than control samples at the same times of study. Fisher test was used to statistically empower our observations, demonstrating a significant variance difference between control and parasite treated RBCs samples ($p < 0.0001$ for both parasites).

These results indicate that there would be an interaction between red blood cells and larvae causing a change in the fractal patterns, which may be associated with the desialization of erythrocyte membrane [4].

Fig. 4 shows in more emphatic form our analysis, which can be clearer when displayed together; both parasites treated are compared with a general control. Evidently there are no visible differences between both controls at the studied times. Moreover, a lowest dispersion for the treated samples compared with controls is undoubtable. In turn, *A. lumbricoides* treated RBCs have a more rapid decrease in fractal slope, while in those treated with *T. spiralis* a nearly constant variation in fractal slopes is observed. These results might suggest a differential action undertaken by both parasites over RBCs.

4. Conclusions

In this report, a novel method to determine the erythrocyte aggregation when incubated with *Ascaris lumbricoides* and *Trichinella spiralis* parasites was developed. The different results on D when studying the samples population could help to establish the type of functional coupling between the RBCs membrane and the larvae parasites.

Little information exists to explain the erythrocytes aggregation process when cells interact with the larvae parasites [14–16]. Present 2D data suggest that the percentage of agglutinates or aggregates shapes, are reflected in the corresponding D measurement values in both *Ascaris lumbricoides* and *Trichinella spiralis* parasites. In this context we considered that the box-counting method could be very useful for developing new approaches as well as elucidating a number of aspects that had not been previously appreciated.

Results showed that *Ascaris lumbricoides* produces increased erythrocyte aggregation, which might correlate with a decrease in fractal dimensions of treated RBCs. In the case of *Trichinella spiralis* there is a constant D value in contrast to a more disperse value showed in controls. It is important to remark that this information cannot be found by linear approach.

The hypothesis that normal samples are represented by more random values (noise) than treated RBCs, where predictability is increased, could be confirmed quantitatively by the estimation of box counting dimension. These preliminary results will be complemented by new experiences.

Certainly, the use of these fractal quantifiers is not intended to replace conventional analysis, but to provide further insights into the underlying aggregation mechanism.

References

[1] Ponce de León P, López Murúa G, Bertorini G, Vasconi MD, Riquelme B. Efecto biorreológico de larvas de Trichinella spiralis sobre la agregación eritrocitaria mediante análisis digital de imágenes. Ciencia y Tecnología. UNR Editora 2013:452–4.

[2] Mukherjee R, Chaudhury K, Chakraborty C. Topological features of erythrocytes in thalassemic patients: quantitative characterization by scanning electron and atomic force microscopy. Anal Quant Cytopathol Histopathol 2014;36:91–9.

[3] Ponce de León P, del Balzo G, Riquelme B. Biorheological action of ascaris lumbricoides larvae on human erythrocytes. Cell Biochem Biophys 2013;65 (2):237–42.

[4] Korol Etchegaray A, Herrera Insaurralde M, Lamónaco AN, Leiva Viú N, Cantalejo Ceccato M, Ponce de León P. Evolución de la dimensión fractal y correlación temporal de agregados eritrocitarios producidos por ascaris lumbricoides. Rev Cubana Med Trop 2013;65(3):283–96.

[5] Imran Razzak M. Automatic Detection and Classification of Malarial Parasite. Int J Biomet Bioinformat 2005(1):1–12.

[6] Rapa A. Fractal dimensions in red blood cell. Turk J Vet Ani Sci 2005;29 (6):1247–53.

[7] Talu S, Stach S, Kaczmarska M, Fornal M, Grodzicki T, Pohorecki W, Burda K. Multifractal characterization of morphology of human red blood cells membrane skeleton. J Microsc 2016;262(1):59–72.

[8] Mandelbrot BB. The Fractal Geometry of Nature. San Francisco: WH Freeman; 1983.

[9] Grassberger P. An optimized box-assisted algorithm for fractal dimensions. Phys Lett A 1990;148(1–2):63–8.

[10] Fernández A, Schuster HG. Deterministic Chaos, VCH Verlagsgesellschaft, Weinheim. 273 Seiten, Preis: DM 108, Berichte der Bunsengesellschaft für physikalische. Chemie 1988;92(9). 1059A-1060.

[11] Falconer K. Fractal geometry: mathematical foundations and applications. John Wiley & Sons; 2004.

[12] Tricot C. Curves and fractal dimension. Springer Science & Business Media; 1994.

[13] G. Vuidel. Fractalyse software. Version 2.4. Available from: <www.fractalyse.org>.

[14] Riquelme B, Rasia RJ. Un material viscoelástico de interés especial: El glóbulo rojo humano. Anales AFA 1997;9:255–9.

[15] Rasia RJ, Porta P, García Rosasco M. Shear deformation measurement of suspended particles. Applications to erythrocytes. Rev Sci Instr 1986;57:33–5.

[16] Riquelme B, Rasia RJ. Complex viscoelasticity of normal and lectin treated erythrocyte using laser diffractometry. Biorheology 1998;35(4–5):325–34.

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