

Increased entropy in *in vitro* activated platelets

G.Bianciardi

Department of Medical Biotechnology, Anatomia Patologica, Università di Siena, Via delle Scotte, 653100 Siena, (ITALY)
E-mail : giorgio.bianciardi@unisi.it

ABSTRACT

Computerized fractal analysis was used in the present work for objective characterization of the entropy of the platelet contour in *in vitro* stimulated platelets by low level thrombin (0.02 U/ml), collected from healthy individuals and observed by means of transmission electron microscopy. Platelet boundaries were extracted by means of automatically image analysis. Information dimension of the platelet contour (measure of the entropy of the platelet outline) was automatically evaluated. The results showed that the platelet boundary observed by electron microscopy is fractal and that, after an *in vitro* platelet activation test, the shape of platelets present increased entropy in comparison to the no stimulated platelets ($p < 0.001$), with 100% correct classification. The entropy of the platelet contour observed by transmission electron microscopy provides accurate, quantitative, data to study platelet activation. The results may play important roles in the evaluation of the platelets status in pathological conditions, like as atherosclerosis and diabetes mellitus, where in *in vivo* activated platelets have been described. ! 2014 Trade Science Inc. - INDIA

KEYWORDS

Platelets;
Fractal analysis;
Information dimension;
Entropy;
Platelet activation.

INTRODUCTION

Among the parameters that born from a fractal analysis, a technique that has become very useful in science^[1-8], the fractal dimension is of great importance in biology and medicine, also contributing to perform diagnosis and prognosis of the patient^[9-19].

We can recall how anatomical entities show complexity as a basic characteristic, residing in the structure and in the behavior of the cell, organ and apparatus^[20]. Matching the variety of the complex natural objects, Benoit B. Mandelbrot created a new language to describe them, the so-called "fractal geometry"^[21]. In particular, where surface phenomena are of crucial importance, a number of complex anatomic structures dis-

play fractal-like properties^[22]. The recent paper by Kraus et al^[23], characterizing the platelets by light microscopy showed that platelets display self-similarity, or, in other words, they are fractals. Here, we tested the hypothesis that the platelet entropy, measure of the complexity of the platelet boundary, observed at high resolution by means of transmission electron microscopy be able to characterize the *in vitro* activated platelets by low level of human thrombin and distinguishing them from resting platelets.

MATERIAL AND METHODS

Platelets

Platelets were collected as Platelet Rich Plasma

(PRP). To obtain PRP, blood samples were withdrawn at minimal stasis from the left antecubital vein of healthy volunteers using a plastic syringe containing 3.8% sodium citrate (1:8) and immediately centrifuged for 15 minutes at 100 g at room temperature in a plastic tube.

In vitro activation study

To perform the *in vitro* activation study, PRP (n=5) was removed with a plastic pipette and 1 ml of PRP was placed into a clean polypropylene vial, immersed in a 37°C water bath, and *in vitro* stimulated by adding 0.02 U/ml human thrombin for 10 minutes at 37 °C (“activated platelets”). Another aliquot of PRP was immediately fixed by glutaraldehyde (“resting platelets”).

Electron microscopy

Glutaraldehyde-fixed platelets were postfixed in osmium tetroxide (1%), dehydrated by acetone, embedded in Araldite and stained with lead citrate and uranyl acetate. Fifty platelets for sample were grabbed at x 3200 without any selection.

Image analysis

Platelets were enlarged to fit into a 500x500 pixel window. By grey level threshold segmentation, single pixel outlines of the contours of the platelets were automatically obtained (JMicroVision 1.27 software: www.microvision.com, Figure 1) and the entropy^[24,25]

of the skeletonized images was automatically measured using the box-counting algorithm (Benoit 1.3 software, TruSoft Int’l Inc: <http://trusoft-international.com/benoit.html>,^[26]).

Entropy (information dimension)

To evaluate the information present in the pattern (entropy), information dimension, a robust estimate from a finite amount of data that gives the probability of finding a point in the image, was calculated. The set was covered with boxes of linear size, d , keeping track of the mass, m_i (the amount of pixels) in each box (from 10 to 100 pixels), and calculated the local information entropy $I(d)$ from the summation of the number of points in the i -th box divided by the total number of points in the set multiplied for its logarithm^[6]. The slope of the log-log plot of information entropy vs. $1/\text{box side length}$ represented the information dimension of the distribution. The methodology was validated by measuring computer generated Euclidean and fractal shapes of known information dimensions. Inter- and intra-observers errors of the entire procedure were < 3%

The log-log plots used to calculate the information dimension showed a straight line with high correlation coefficient, always above a value equal to 0.99, thus justifying the fractal approach (Figure 2).

Statistical analysis

The Kruskal-Wallis test was applied in order to verify significant differences between the groups. A linear regression analysis was applied in order to verify the significance of the log-log plot. In order to evaluate the predictive significance of fractal dimension with respect to the subjects a chi-square test was applied to the grouped cases classified by entropy (D1 cut-off = 1.13) according to the activation status.

RESULTS

Transmission electron microscopic examination of the *in vitro* activated platelet samples revealed morphologies characteristic of a state of platelet activation: organelles clumping into the center presence of extended pseudopodia (the so-called “platelet shape change”) (Figure 1, bottom left).

Running the automatic fractal analysis software, the log-log plots reveals that the platelets, resting or activated platelets, are fractals when observed in transmis-

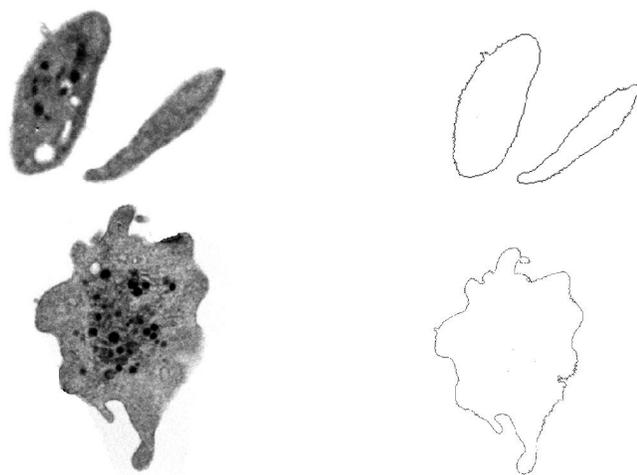


Figure 1 : Transmission electron microscopy of platelets (left) and their contours after thresholding (right) in no stimulated (top) or in vitro stimulated samples (bottom). Note that in the activated platelet, the platelet organelles clumping into the center of the cell and long pseudopods are present, aspects of the platelet shape change. Original magnification, x 3200.

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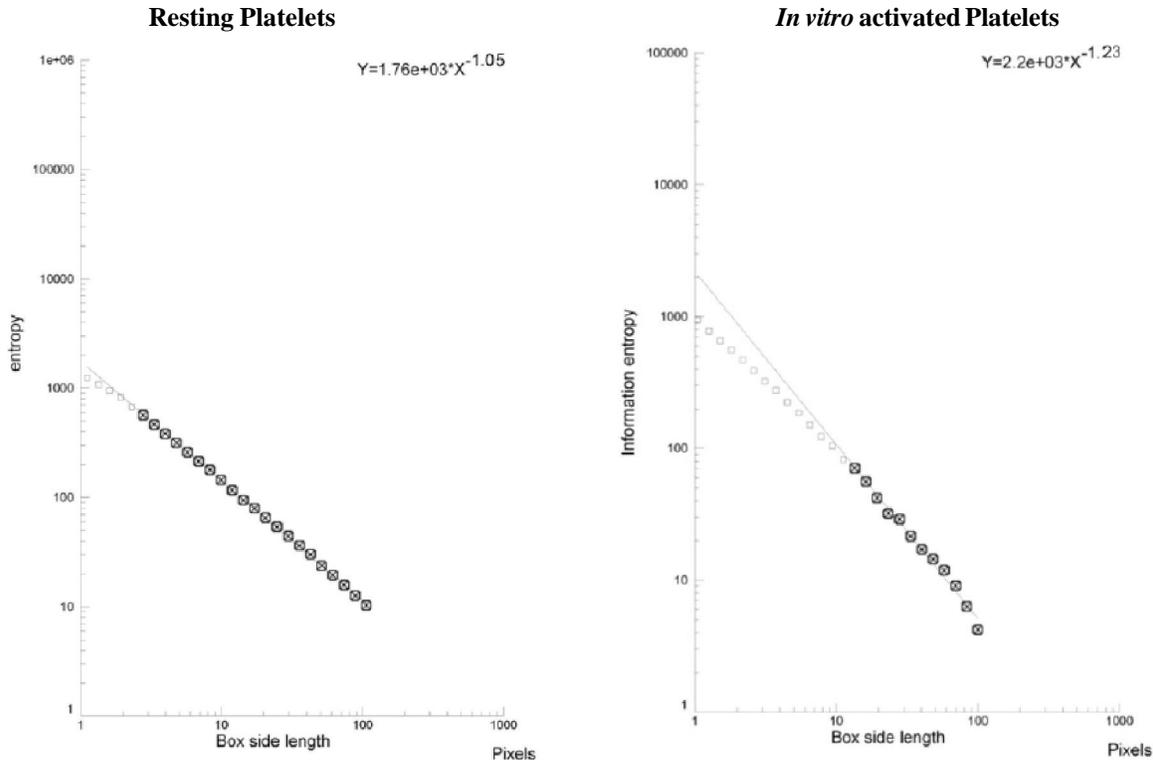


Figure 2 : Log-log plots of the single-pixels outline of platelets, no stimulated (left) or in vitro activated by low level thrombin (right). The slope (the exponent of the log log straight line) is the entropy of the platelets at the chosen scales. The linearity of the log-log plots ($p < 0.001$) indicates that, in the scaling window used, the platelets are self-similar, or “fractals”, when observed by means of transmission electron microscopy. Note that in vitro activated platelets (right) present a higher values of entropy than the resting platelets (left).

In vitro activated platelets vs resting platelets

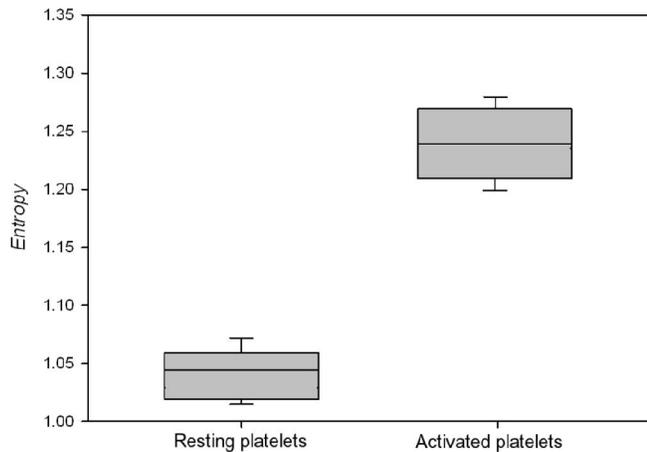


Figure 3 : Box Plot, mean + outliers (5th and 95th percentile). Platelet Entropy: no activated platelets (resting platelets) vs. in vitro activated platelets, $p < 0.001$

sion electron microscopy, as evidenced by the linearity ($p < 0.001$) of the log log plots (Figure 2).

In vitro activated platelets present higher values of entropy than no activated samples (resting platelets) ($p < 0.001$, TABLE 1, Figure 3).

The percentage of grouped cases classified by the

TABLE 1 : Entropy of the platelet contour in healthy subjects. Platelets were in vitro stimulated by 0.02 U/ml human thrombin (“activated platelets”) or not stimulated (“resting platelets”). Mean values \pm standard deviation.

	Entropy \pm S.D.
Resting platelets (n=5)	1.05 \pm 0.02
<i>In vitro</i> activated platelets (n=5)	1.24 \pm 0.02
p	< 0.001

In vitro stimulated platelets present entropy values higher than the one of resting platelets.

entropy of the platelet contour (cut-off = 1.14) according to the state of activation showed a 100% ratio between the number of correctly classified cases and all cases, $p < 0.001$ (TABLE 2).

DISCUSSION

The term fractal is a geometric concept related to highly irregular shapes, with non-integer, or fractional, dimensions, and a property known as self-similarity^[21]. Unlike a smooth Euclidean line, a theoretical fractal line, which has a dimension between 1 and 2, is irregular or

TABLE 2 : Predicted group membership by fractal analysis, resting platelets vs. *in vitro* activated platelets

Actual Group	No. of cases	Predicted group membership	
		Resting	Activated
Low Entropy	5	5	0
		100%	0%
High Entropy	5	0	5
		0%	100%

Percent of grouped cases correctly classified by the information dimension (entropy, cut-off= 1.14), according to the status of platelet activation: 100% ratio between the number of correctly classified cases and all cases, $p < 0.001$, $p < 0.001$.

wrinkly. Examination of these wrinkles with the lens of a microscope reveals smaller wrinkles on the larger ones. Further magnification shows yet smaller wrinkles and so on. A natural fractal, an object composed of subunits that resembles the larger scale structure, so maintaining the same, at least statistically, fractal dimension (self-similarity or scale-invariant property), is present in a variety of biological structures: cardiopulmonary structures and the ramifying tracheo-bronchial tree^[22], the His-Purkinje network and the cardiac muscle bundles^[27] as well as the placenta's arterial tree^[28]. The meaning of these fractal structures in the human body is profound. The self-similar tracheo-bronchial tree provides an enormous surface area for exchange of gases at the vascular-alveolar interface, coupling pulmonary and cardiac functions^[21]. For the vasculature, fractal branching provide a rich network for distribution of nutrients and oxygen, as well for the collection of metabolic waste products^[22]. A variety of other organ systems contain fractal structures that serve function, e.g., the fractal organization of connective tissue in the aortic leaflets that relates to the efficient distribution of mechanical forces^[29].

In effect, network structures and scaling laws developed in broad, quantitative, mathematical approaches must to be characterized in medicine to understand health and disease^[30]. If we follow the strict criteria of a fractal window, where biological components are statistically self-similar, the so-called "scaling windows", or, in other words, within upper and limits of magnification, we can depict complex biological shapes as fractal entities characterized by a nonlinear behavior^[31]. In effect, fractal analysis is emerging as a powerful tool to perform differential diagnosis^[9-19] and prognosis of the patients in cancer^[32] or to follow an-

ticancer therapies^[33].

In the present paper, fractal analysis has been tested in order to search for a different entropy of the platelet contour in *in vitro* activated platelets in comparison to no activated (resting) platelets by using transmission electron microscopy, in order to have a high resolution of the cells. We show that the platelet contour observed in transmission electron microscopy is fractal and that *in vitro* stimulated platelets present higher values of entropy than in resting platelets (healthy subjects).

In this work, entropy appear as accurate descriptors of the platelet shape-change upon *in vitro* platelet activation by low level of human thrombin, also in agreement of the results obtained in light microscopy by M-J Kraus, using other platelet agonist, like ADP and TRAP and different fractal approaches^[23].

CONCLUSION

The evaluation of the entropy of the platelet contour was able to distinguish accurately between activated platelets vs resting platelets, with a 100% correct classification, giving us a new approach to objectively and accurately quantify the status of platelets.

This method may be promising to study circulating platelets in pathophysiological condition linked to the *in vivo* platelet activation (atherosclerosis, diabetes mellitus and so on) and after administration of drugs or other therapeutic procedures.

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