

VIDEO RECORDING AND TIME RESOLVED DIGITAL PARTICLE IMAGE VELOCIMETRY IN EVALUATION OF ANTIAGGREGANT DRUGS

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Manuscript received: September 2015

Abstract

This paper presents an analysis of erythrocytes aggregation and sedimentation processes, using three methods: (i) the sedimentation curve determined by measuring the height of sediment, (ii) the video observation and recording of microscopic images during sedimentation (iii) the analysis of digital particle image velocimetry (PIV) as mathematical method for the estimation of red blood cells aggregates trajectories and velocities. The main finding was the co-existence of ascendant and descendent flow tubes. Single erythrocytes and small aggregates appeared to move up as a counter stream coupled with descending flow of greater aggregates. The mathematical analysis revealed an even more complex picture, including turbulences and formation of local vortexes. Since our experiments with clopidogrel proved an effect of decreasing erythrocytes sedimentation, the PIV method was further applied in analysis of samples containing 4 µg/mL drug. It was observed a significant change in the distribution of speed velocities.

Rezumat

Articolul prezintă analiza agregării și sedimentării eritrocitare folosind trei metode: (i) determinarea curbelor de sedimentare prin măsurarea înălțimii sedimentului, (ii) observarea și filmarea de imagini la microscop a tuburilor de curenți formați din agregate eritrocitare din procesul de sedimentare (iii) analiza digitală a vitezei particulelor folosind modele matematice. Au fost observate agregate eritrocitare mari cu o deplasare descendentă și agregate eritrocitare mici cu o deplasare ascendentă sub forma unui contra-curent. Analiza matematică a pus în evidență un tablou și mai complex, incluzând turbulențe și formarea de vârtejuri locale. Experimentele noastre au demonstrat că antiagregantul plachetar clopidogrel are un efect de scădere a sedimentării eritrocitare. Mai departe metoda de analiză matematică a imaginilor (PIV) a fost aplicată pe probe de sânge tratate în prealabil cu 4 µg/mL clopidogrel. S-a observat o schimbare în distribuția traiectoriilor vitezelor vectorilor asociați eritrocitelor din imagini.

Keywords: erythrocytes, aggregation, particle image velocimetry, clopidogrel

Introduction

The Erythrocyte Sedimentation Rate (ESR) is a simple test for assessing the inflammatory status [34, 39]. ESR has also been found to be clinically significant in the evaluation of non-inflammatory diseases such as prostate cancer [16], coronary artery disease [9] and stroke. In combination with other clinical endpoints, ESR proved to be useful in predicting the evolution of short-term stroke [4]. Aggregation of red blood cells (RBC) is a major determinant of blood viscosity and blood circulation through vessels with slow flow [20]. ESR is an important parameter for diagnostic, for example in differentiating active Crohn's disease from intestinal lymphoma, intestinal tuberculosis and Behcet's syndrome [21]. The International Committee for Standardization in Haematology (ICSH) recommends the use of Westergren method for evaluating the sedimentation process [15, 46]. The single-parameter

used to characterise the erythrocyte sedimentation by ESR represents a very crude approach of complex phenomena involved in erythrocytes aggregation and sedimentation [1, 2, 25]. There were developed many models for aggregation of blood platelets based on the description of aggregation dynamics. Practically all aggregation dynamic models are based on the extended Derjanguin-Landau-Verwey-Overbeek (xDLVO) theory, applied initially to colloidal particles, and later to micro- and nano-drug delivery vectors [8, 27, 30, 48]. This theory proved to be appropriate also for describing aggregation of cellular systems, viruses, plasma proteins, etc. [12, 14, 24, 57, 58]. The proposed models were in connection mainly with platelet aggregation, but the results may be extended to erythrocytes aggregation process also. Models concerning kinetics of aggregation started from the hundred years old theories of von Smoluchowski

and Stokes sedimentation law for spheres in viscous fluids and were actualized later in the frame of population balance models [26, 31, 48].

The present paper is an experimental evaluation of the blood sedimentation using video recordings. It was proposed a mathematical approach for the local simultaneous evaluation, in microscopic domains of the aggregation and flow of erythrocytes and of erythrocytes complexes. The video methods were recently applied for the evaluation of blood cell aggregation [41]. Due to similarity between the platelet and erythrocyte aggregation mechanism, it was expected for the antiaggregant drugs to reduce both erythrocyte aggregation and sedimentation. The final goal of the new method was to propose an alternative to the direct study of drug effect on platelet aggregation.

Particle Image Velocimetry (PIV)

Cross-correlation is a measure of similarity of two time series as a function of the time lag of one image relative to the other.

For two continuous functions f and g , their cross-correlation is defined as:

$$f * g(\tau) = \int_{-\infty}^{\infty} f(t)g(t + \tau)dt$$

where T is the considered time interval between selected images.

In particular, for discrete functions, the cross-correlation becomes:

$$f * g(n) = \sum_{m=-\infty}^{\infty} f(m+n)g(m)$$

The cross-correlation of two functions is similar to their convolution.

$$f * g(\tau) = \int_{-\infty}^{\infty} f(t)g(t - \tau)dt$$

A useful property is the fact that "Fourier Transform" of the convolution product of two functions equals the product of their transformations

$$F(g * h) = F(g)F(h)$$

In probability and statistics, the definition of correlation includes additionally a normalizing factor in such a way that correlations have values between -1 and +1.

Particle image velocimetry (PIV) method selects "interrogation" areas from two successive images obtained at a time interval τ and using the correlation function, matches the points and calculates their dislocation in space. The ratio between distance and time interval defines the velocity of corresponding particle. PIV is

considered to be a reliable non-invasive method being used in many domains such as aerodynamics, hydraulics, biology systems, etc. This technique can reveal two-or three-dimensional (2-D or 3-D) flows with high accuracy.

Difficulties in practical applications arise from the necessity to detect and remove outliers by successive application of "smoothing" algorithms [13, 19, 22, 33, 38, 55].

In case of successive images of blood, cells or cells aggregates, all together can be considered as "particles". In diluted samples, before the aggregation reaches a significant threshold, it is expected that the method will work well enough. Further, in concentrated samples, the differences between the size of aggregates, as well as the possibility of aggregating or disaggregating in the time interval between images could bring artefacts and significant difficulties in analysis.

Materials and Methods

Global evaluation of the reversibility of aggregation

"Moving boundary" represents the moving of the demarcation line between clear blood plasma and erythrocyte sediment. The speed of the "moving boundary" for each sample was determined over one hour interval. In order to test the reversibility of aggregation, sedimentation and the uniformity of compactness, after one hour from the first reading, the samples were stirred for two minutes and the sedimentation curves were determined again (data not presented in graphical form). The experiment was performed in parallel for five samples, repeated five times without interruption between determinations and after 24 h. Four parameters of the curves were compared: the lag-time, the slope of linear part, the maximum height of sediment and the area under sedimentation curves $AUSC_{0-1h}$.

Local evaluation of cell movements and cell aggregates

Experimental device. In order to evaluate the local aggregation and the sedimentation processes, a special experimental device was built (Figure 4).

Blood Sample preparation. Blood samples, obtained from volunteers in a time frame of maximum four hours from collecting, were centrifuged at 100 G in plastic tubes on anticoagulant. Before participating in this study, the volunteers gave their informed consent. Red blood cells were separated from plasma poor cells and were kept in capped plastic test tubes. Different dilutions of red blood cells in plasma were obtained by a mixture of plasma and red blood cells.

Video image processing. Local processes in the erythrocytes suspension populations were visualized using a special designed device comprising an IOR

ML-4M microscope with a 16 Mp camera sensor for video recording. The recorded film was sequentialized in stacks of individual images at a rate of 27 frames per second. Each stack of images was analysed using the PIVlab Program methods from the mathematical software MatLab [41-44].

Statistical evaluation. One-way ANOVA was applied to compare the variance of parameters within groups of determinations and the variance between means of successive determinations, in order to test the hypothesis that the parameters curves corresponding to successive determinations are equal.

Computerized evaluation. It was used the PIVlab application software, working in MatLab, purchased from MathWorks, Natick, Massachusetts, USA.

Results and Discussion

Reversibility of aggregation and sedimentation

The sedimentation curves had an S-shape, corresponding to three phases- a time-lag, a movement with constant rate and a saturation phase. Analysis checked for any statistical significant change in the mean of AUSC values. The obtained value for *F* test comparison of Mean Square due to regression and Mean Square Error indicated that, for $p < 0.05$, the slope is zero, and data differ only following a random error. The findings of the statistical and phenomenological analysis were:

- the existence of a clear boundary and the linearity of its movement indicates a narrow distribution in the speed of aggregates and further, a very possible narrow distribution in the size of aggregates;
- the aggregation and compactness of sediments are completely reversible;
- the constancy in time of all parameters of the curves suggests that the entire set of phenomena connected with aggregation and sedimentation the same within the series of consecutive determinations. Therefore, the global approach suggests the following picture for "sedimentation". At the beginning, in the first short time interval, aggregation runs very quickly and incomplete, i.e. a stationary state of equilibrium between aggregation and disaggregation and an approximately constant size of aggregates was noticed. After aggregation, sedimentation proceeds with constant velocity. Finally, the sediment opposed resistance to further sedimentation.

Part of these observations and the above mentioned hypothesis occurred from the first sedimentation experiments, performed hundred years ago. Our comparison of sedimentation curves instead of usual two points, in repeated experiments on the same samples, offers more reliable arguments for what was intuitively felt during the time.

A series of experiments (data sent to be published separately) proved that platelet antiaggregant

clopidogrel, in the concentration range of 1 - 8 mg/L, decreases sedimentation of erythrocytes. The effect was also reversible and reproducible.

Microscopic evaluation.

Aggregation and size distributions.

It was evaluated the size of aggregates in diluted and in concentrated blood samples. In very concentrated samples (undiluted or moderately diluted blood), appeared large domains of loosely bound Red Blood Cells (RBC), fluctuating between aggregated and disaggregated state, fragmented by small plasma domains, void of RBCs or containing only singular RBC (Figure 1).

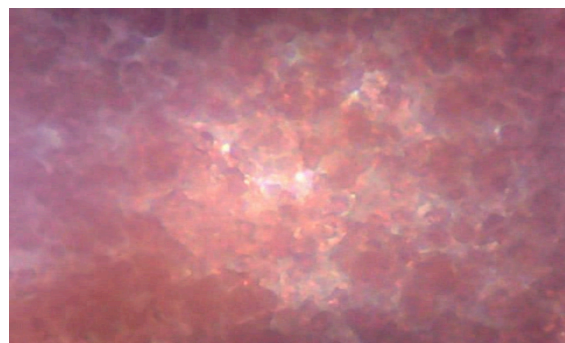


Figure 1.

Partition of RBCs suspension in different fields

In diluted samples, it was observed that size distribution is, in spite of Smoluchovsky's equation predictions, rather bimodal: there are single erythrocytes or large erythrocytes complexes with only a few intermediary size. Doublets and triplets were not observed.

Smoluchowski equation [40] was fit to describe the variation of erythrocyte aggregate size in time. The problem is that the equation predicts at infinite time the aggregation of all cells to a single one, huge aggregate. In order to remove this contradiction, it was considered that aggregation is counterbalanced by dissociation [56].

The fact that in vascular tree, in normal conditions, aggregation is absent or reversible, is largely accepted, because of shear stress in large vessels, which induces disaggregation of aggregates formed in small vessels. In shear flows, a rouleaux-like aggregate will be fragmented [56]. If adding a new term in the equation, accounting for disaggregation, does not present difficulties, the solving of resulted equations and obtaining an analytic solution is an infinitely much complex, even impossible problem. Numerical solutions and identification analysis of the deduced non-linear differential equation for the size of aggregate were assessed [29]. Alternatively, increased ESR has been attributed to increase amounts of some plasma proteins, mainly fibrinogen. Several authors [3, 5, 6, 10, 28] quantified the surface affinities of erythrocyte

membranes for macromolecules and related them to aggregation, but, as a rule, there is a very poor quantitative correlation between ESR and fibrinogen concentration.

During our experiments fibrin bridges between RBCs were not put in evidence.

Sedimentation. The method demonstrated that the sedimentation process is achieved through a complex

mechanism. Although the global evaluation revealed an overall sedimentation process, at local level the process was not unitary anymore, both descendant and ascendant streamlines being observed. Particularly, it appeared that the descendant flow was mainly connected to larger size erythrocyte complexes.

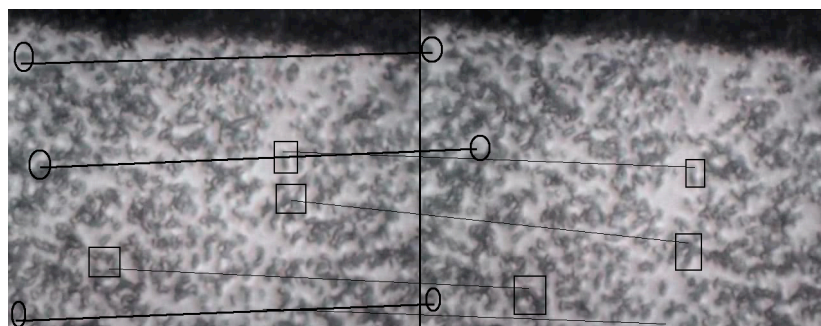


Figure 2.

Descendant movements of aggregates and ascendant movement of RBCs

The ascendant flow is concerned mainly to plasma mixed with single erythrocytes or small erythrocytes complexes. At some points, there were bridges between the two main types of flows and swirls were formed. Recently it was considered from the fluid mechanics point of view that red blood cells aggregates deform and migrate within vessels, forming heterogeneous distributions. This has a considerable effect on local hemodynamic [37]. Some authors tried to explain the nonlinearity by considering blood as a three phases system: plasma, cellular components and gas in the form of nano-/micro-bubble [49-54]. In our experiments air bubbles appeared only in the case of closed cuvettes. Sedimentation of single erythrocytes, even in absence of plasma ascending flow, is extremely slow. The first problem is that blood is an extremely concentrated suspension (5 million of RBC/mm³), so that motions are highly restricted and influenced by interactions between cells. A high non-linearity of the motion appears [18, 49]. Einstein investigated the disturbances caused by a particle suspended in a flow with a constant velocity gradient [11]. He proved theoretically that a viscosity increase of a fluid carrying solid particle is connected to the volume fraction of the particles by the law:

$$\eta = \eta_0(1 + 2.5\varphi)$$

where η_0 represents the viscosity of the fluid, and φ is the concentration of particles. The problem is that his theory was conceived for much diluted suspensions, being not applied for blood.

Design of experimental device for video image processing

Previous attempts to analyse erythrocytes aggregation process at microscopic level were made using a regular photomicroscope in a regular configuration, with glass cuvettes and high dilutions of blood. It was left to sediment in cuvettes in a vertical position and at given times, they were placed on the horizontal table of the microscope to be analysed [36]. This procedure gave good results in making different snapshots of the process without capturing the entire process.

In our experiments, the first step in making a good analysis was to find the most appropriate cuvettes. Trials have been made with different types of cuvettes, test tubes, micro-haematocrit, and square cuvettes. It has been observed that the best way to produce decent microphotographs of blood sedimentation was using square cuvettes. Round glass tubes are almost impossible to observe at microscope because of light refraction. Light exits at different angles because of the round passing through the glass walls and produces a deformed image in which the process cannot be observed.

Different attempts on producing accurate blood sedimentation cuvettes where also made. First experiments consisted in designing rectangular cuvettes by bonding glass slides pieces and polycarbonate edges using cyanoacrylate resin. Resulting cuvettes had interior dimensions of 50 mm height, 2 mm wide and 1 mm thickness. In spite of giving similar results as Westergren method in ESR measuring, this kind of cuvettes had a major downfall. Blood layers are too thick, so normal microscope light sources can't penetrate the sample. Increasing the power of the light sources

would eventually result in rising temperature of the slides generating Brownian vortex. The best thickness appeared to correspond at a blood layer of approximately 0.3 mm.

Rectangular cuvettes from glass slides with an interior gap thickness of 0.3 mm had the benefit of using small quantities of blood per analysis. The blood is sucked into the cuvettes by capillarity pressure and no additional pipettes are needed. One possible drawback of this kind of cuvettes is the small quantity of air remained trapped in the glass cuvette when the blood is sucked in. Removing this air is very difficult.

Different types of experiments were conducted in order to resolve this problem and we came up with two different designs. The first design consisted in two glass slides bonded together with two vinyl inserts making a rectangular tunnel in which blood is sucked in and stays without dripping out.

The second design was more efficient as it was comprised by using two glass slides bonded together with a “w” shape insert. This design is more efficient because it allows the air to escape from the bottom of the cuvettes and having just a single side opened, is less hazardous for the operator. Examples of those different designs are shown in Figure 3.

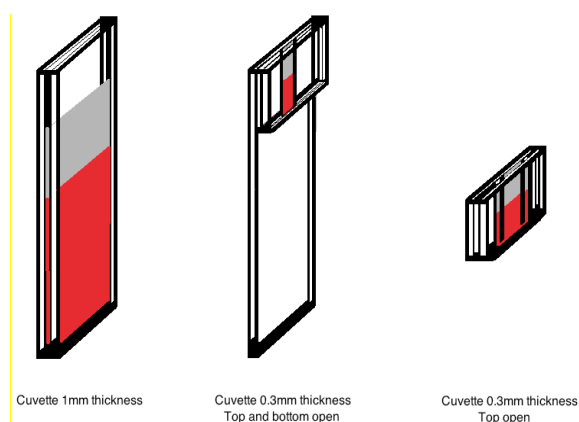


Figure 3.

Design of experimental cuvettes

The most crucial step in making a continuously analysis of erythrocyte sedimentation is the ability to observe the process in normal conditions. It was concluded that the best way to perform the experiments was to put the sample in vertical position and adapt the microscope.

An IOR ML-4 microscope especially modified was used. A vice mount mechanism in order for the microscope to stay in a rigid and stable horizontal position was used. This way the samples stayed upright. The power source was replaced with a powerful 12v, 10w, COB LED light. It resulted a monochromatic white light, very bright and colder than the regular, original incandescent 60w 12v

light bulb. This meant that less aberrant blood stream movement was avoided.

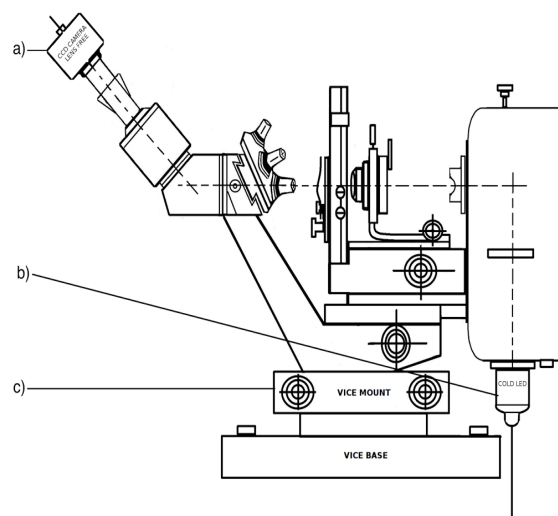


Figure 4.

Experimental microscopic device (a) Camera sensor, (b) cold light source, (c) vice mount

The film captured the process of erythrocytes aggregation with the help of the microscope objective 4 x, 10 x and 20 x, taken from the layer below the demarcation line of the sedimentation process. The whole process had duration of 120 seconds.

The film was dissembled in individual pictures with a distance of one second (1 frame of 27) from each other. A succession of film images was analysed with the PIVlab Program from Mat Lab.

The images were processed in order to increase the contrast and to remove the aberrations. In PIV technology, it is generally desirable to reduce the amount of erroneous velocity estimation by enhancement of images before the actual image correlation takes place [4].

Particle Image Velocimetry

Particle image velocimetry is a general mathematical method for comparison of time changes of images. The software, after comparing of successive images, calculated the velocities of particles using Fast Fourier Transform (FFT), which further can be represented as vectors, as can be seen in Figure 5.

The vectors stand for the displacement that took place from a position of the particle from the first picture to the last picture from the stack of images captured at a speed of 27 frames/second. Using the images captured, we can see the particle movement in three dimensions: particles that move up and down, left and right, back and forth. Between those directions, the formation of vortices can be observed.

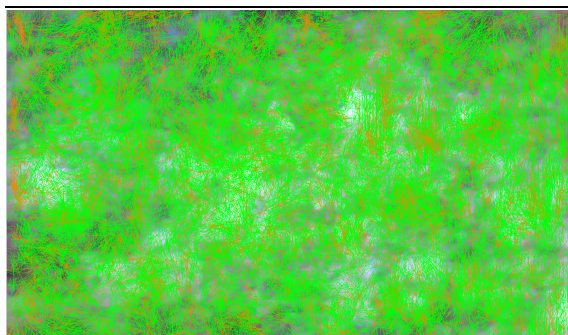


Figure 5.
Velocity vectors identified by PIV analysis

Related with our interest in correlating of local analysis with global observed erythrocytes sedimentation process, we focused on the statistical evaluation on the up and down particles movements. Coordination system was fixed to consider downward positive and negative direction, vertical direction as negative.

The software analysis of the particles vertical movements was performed in two steps. First step comprised the particles speed magnitudes analysis and their directions. Figure 6 shows the scatter plot for the particles movements in the vertical and horizontal plane of the capture, and the histogram plot of the particles speed magnitude.

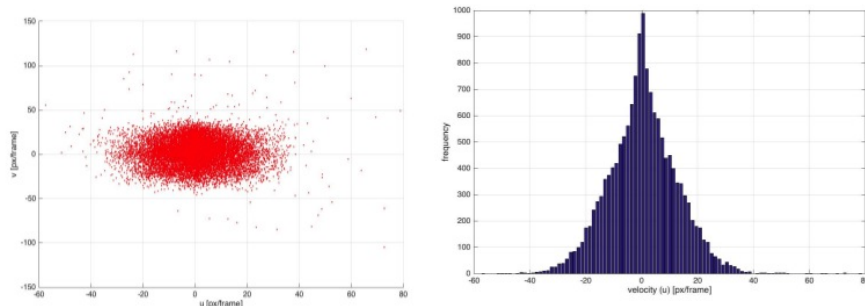


Figure 6.
Scatter plot and histogram of scalar velocities and their frequency

The second step comprised the vectors validation with speed values in specific clusters. That excludes all vectors associated with particles having speed magnitude and movement outside specified ranges. For example validating the vectors with speed values in the domain $[-10:10] \times [0:10]$, it remained for analysis only those vectors associated with particles that move downward with a moderate speed. We identified that those particles were aggregates of erythrocytes moving down. On the other hand, validating vectors with speed values in the domain $[-10:10] \times [0:-10]$ was equivalent

to an analysis of only vectors associated with particles that move upward. We considered that those particles were aggregates of erythrocytes moving up.

The software performed further a second order analysis, clustering the vectors associated to individual particles (STREAM line tool) in tubes containing approximately parallel flow lines. In Figure 7 the stream lines corresponding to clusters of sedimenting particles can be seen.

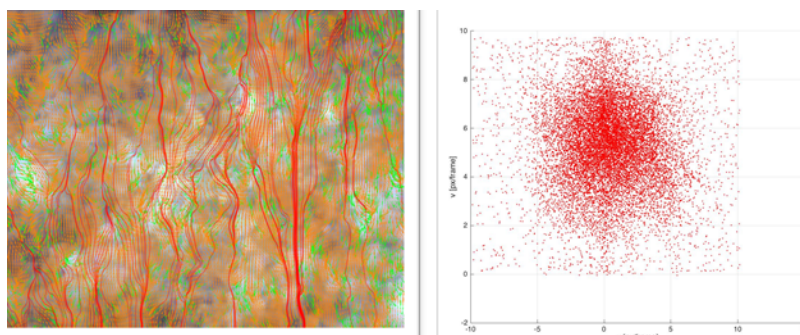


Figure 7.
Stream lines flow tubes and scatter plot velocity of the erythrocytes assemblies in sedimentation process

The sedimentation appears as a large collection of separated tubes of flow. The same pattern appears to characterize the ascending movement of small aggregates presented in Figure 8.

Finally it is remarkable that the evaluation of this multitude of local currents does not suggest the “mass quasi-uniform sedimentation” appearing at global evaluation. However, the positive result of

these local evaluations is, in fact, that disaggregation can be understood as the consequence of the shear

stress over aggregates between the opposite flows of fluid and particles.

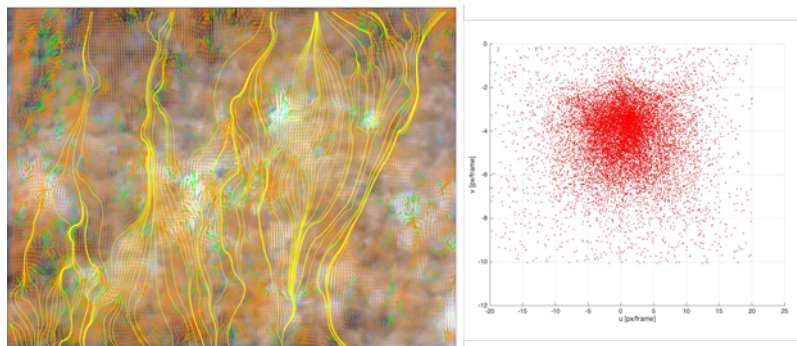


Figure 8.

Stream lines flow tubes and scatter plot velocity of the erythrocytes with an upward motion

Evaluation of samples after addition of clopidogrel 4 mg/L reveals changes in size and distributions of aggregates and in their velocity vectors distributions, as can be seen in Figure 9, with a narrowing trend

for the Gaussian distribution of the aggregates velocities, apparently due to the decrease of the aggregation process.

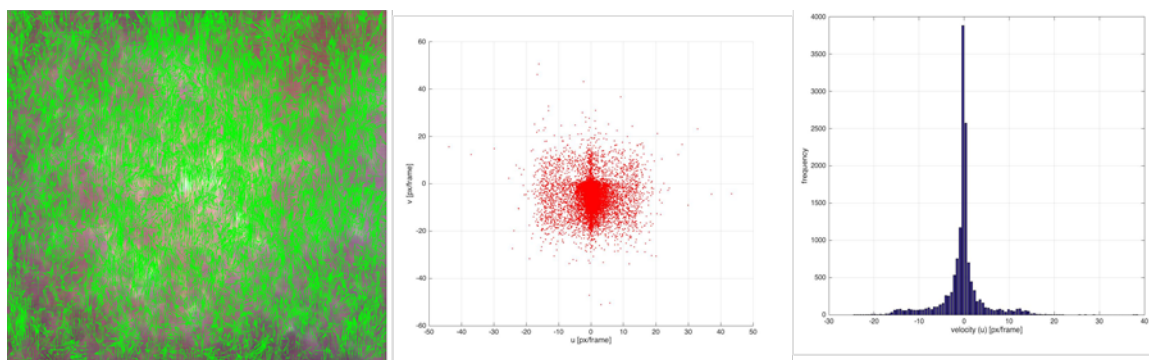


Figure 9.

Distributions of velocity vectors and their magnitudes, after addition of clopidogrel

Interpretation of these changes requires much more and diversified experiments, in a physio-pathological context, being the subject of future research.

Conclusions

The essence of mathematical and experimental approaches of aggregation and sedimentation presented in this paper is the transfer from a global to a local geometry. Although bulk changes in red blood cells suspension looks simple and well characterized, local distribution, until now generally overlooked, suggests infinitely more complex phenomena.

Erythrocytes sedimentation can be characterized globally by the moving of boundary between poor erythrocytes plasma and rich erythrocytes plasma. In order to explain sedimentation it is necessary to suppose that in a first, rapid phase aggregation and disaggregation occur, but the later one cannot be globally put in evidence.

Simultaneous occurring aggregation and sedimentation can be observed and analysed using alternative

methods for evaluation of many, successive images. Video recording proved to be an adequate method for such type of analysis. Since the number of erythrocytes is around five millions/mm³, advanced mathematical and statistical methods are required.

The main finding of the microscope visual inspection was the co-existence of ascendant and descendent flow tubes. Computerized analysis of video images series reveals that aggregates are really shared into two main populations, characterized by the sign of vertical component of their velocity vectors. The long series of local observations and their time course analysis does not reveal the stationary state behaviour of blood sedimentation observed at a global level, but provided an explanation of disaggregation as a consequence of local opposite flows of plasma and aggregates.

This technique of microscopic recording and PIV analysis was used for determining physio-pathological modifications and the effect of drug substances on aggregation and erythrocyte sedimentation. There were observed significant

modifications in the distribution of velocities, but their profound understanding implies extensive further research studies.

Acknowledgements

This paper is supported by the Sectorial Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/137390/..

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