QUANTIFICAÇÃO E TÉCNICAS DE MEDIÇÃO DA AGREGAÇÃO E DEFORMABILIDADE ERITROCITÁRIAS / QUANTIFICATION AND TECHNIQUES OF MEASUREMENT OF RBC AGGREGATION AND DEFORMABILITY

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ABSTRACT

The mechanisms of the reversible RBC aggregation and RBC deformability and the experimental techniques and methods for their quantification are overviewed in the present paper. Their role for the non-Newtonian rheological properties of blood and blood flow are discussed. The clinical symptoms resulting from the increased erythrocyte aggregability are considered.

Key-words: RBC deformability, RBC aggregation, quantification and techniques

DEFORMABILITY OF ERYTHROCYTES

Blood is non-homogeneous system composed of deformable cells suspended in plasma. The red blood cells (RBCs) (40-46% of the blood volume) are much more flexible than white blood cells (WBCs) are easily deformed in flow not only in small vessels but also in large vessels. White blood cells (only 1.2% of the blood volume) could be divided into two groups: monocytes with diameter 16-22 μm and lymphocytes and granulocytes with diameter 10-12 μm. Their role in the immune system is due mainly to their ability to move alone with velocity $(0.6\text{-}7).10^{-7}$ m/s[25]. The number of erythrocytes in unite blood volume (1 mm^3) is about 5.10^6, or about 50 times more than the number of platelets and leukocytes in the same volume -$(4\text{-}10).10^3$. In human blood plasma is 90% water by weight, containing 7% plasma proteins, 1% inorganic and 1% other organic substances. Its viscosity is between 1.2 and 1.4 mPa.s.

The blood cells are undergone to deformations in flow, changes in the curvature and shearing of their components. During this process, the cells are completely adapted to flow, rolling around the cytoplasm and keeping a stationary orientation. Therefore, for normal cell functioning in vivo the deformability of blood cells is crucial. With the absence of nucleus, the low cytoplasmic viscosity, viscoelasticity of its membrane and

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high ratio of surface area to volume, giving an excess area of about 40%, the erythrocytes are ideally suited for the circulation.

Molecular Basis of Erythrocyte deformability

The basic structure of a unit membrane consists of two layers of phospholipid molecules (bilayer) with their hydrophilic heads facing outward and hydrophobic tails locking in the interior by hydrophobic forces[15] (Figure 1). This is also consisting of the intrinsic and extrinsic proteins. The intrinsic proteins are integral part of the lipid bilayer of phospholipids. The skeletal proteins form a network of proteins consisting of spectrin, actin, protein 4.1 and adducin. The underneath spectrin network provides structural rigidity and support for the lipid-protein layer of the membrane.

A conceptualised view of the reversible deformation of the membrane with a change in geometric shape but at constant surface area includes the rearrangement of skeletal network. In this process certain spectrin molecules becomes unfolded and extended while others are more compressed and folded. With the increased deformation the spectrin molecules attain their maximal linear extension. This is the limit of reversible deformation. Beyond this point with the further increase in surface area with application of force the braking of the functional complexes may take place. Thus a combination of lipid bilayer and proteins provide a specialised permeability barrier functions and integrity to the erythrocyte membrane, respectively[42].

Determinants of erythrocyte deformability

It is shown that qualitative and quantitative changes of erythrocyte shape influence the erythrocyte deformability. One important quantitative factor of the erythrocyte shape is

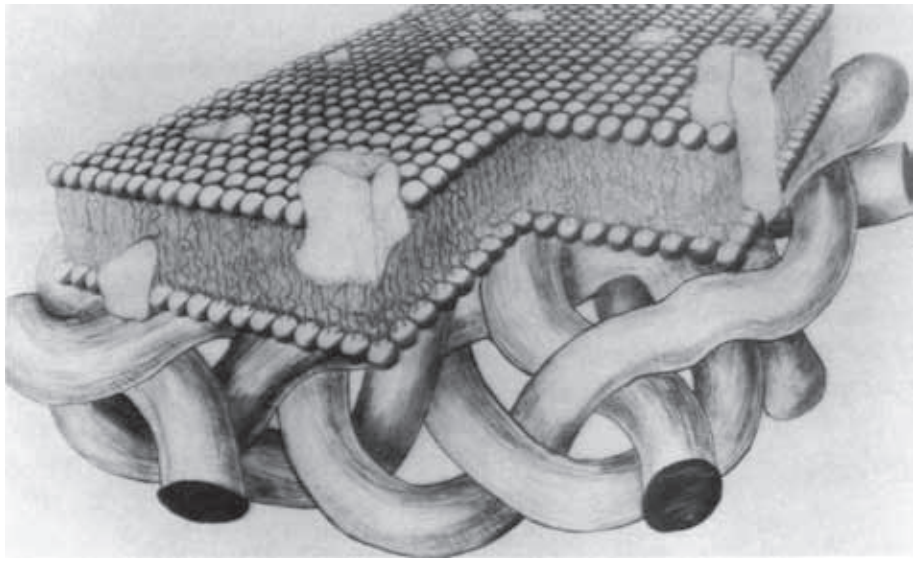


Fig. 1 – An idealized view of the red blood cell membrane, presented by Evans and Skalak (1979) (From Y. C. Fung [16])

the negative curvature of erythrocyte central part. The lowering of gradient of drop in optical density of erythrocyte central part is connected with decrease erythrocyte ability for deformation. Alterations in shape of erythrocyte are often associated with loss of deformability and observed under thermal stress, oxidant stress and prolonged exercise. Immobilization stress induced the changes of RBC morphology and partly caused alterations in normal discocyte form[12]. The measurement of RBC deformability is carried out by means of viscometry, micropipette aspiration, filtration techniques and ektacytometry. The results of the measurements of red blood cell deformability in whole blood and density-separated RBC, determined in individuals in different age groups suggest that, RBC circulating in the vasculature of "aged" individuals exhibit more pronounced rheological alterations, during the aging process of RBC [44].

The deformability of the erythrocyte is dependent on the viscosity of the inner solution within the membrane, which contents in norm is about 33 g% hemoglobin. The viscosity of the inner solution is about 6 mPa.s, but it depends on the physicochemical contents of hemoglobin. The role of the presence of intracellular adenosine triphosphate (ATP) on maintenance of shape and deformability of human red blood cells is determined. Results indicate that deformability is independent of the intracellular level of ATP and only dependent on the shape of the cells[15].

RBC AGGREGATION

Mechanisms of erythrocyte aggregation and different types of interactions

Erythrocyte aggregation is a fundamental parameter of the rheological

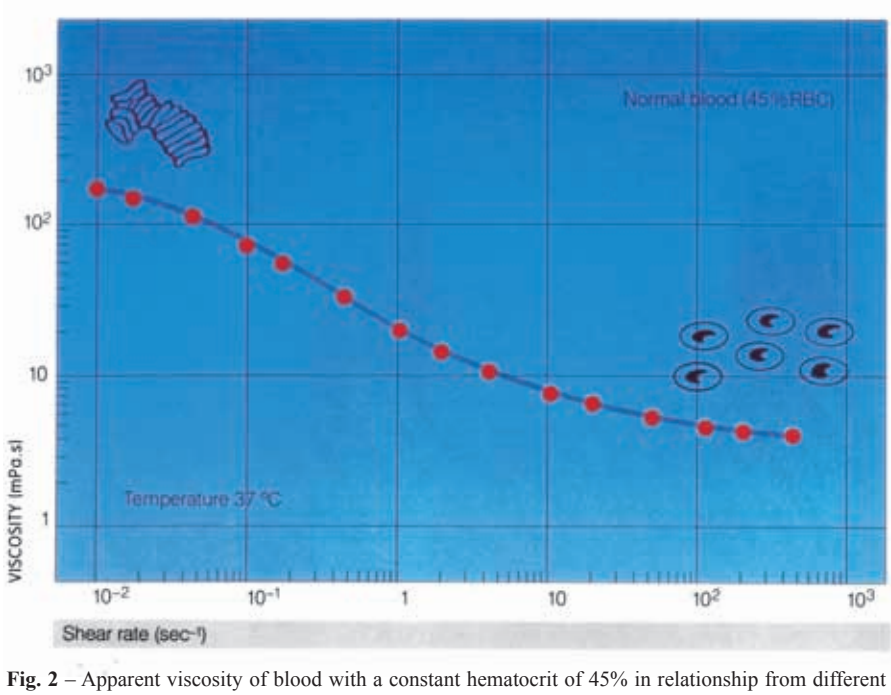
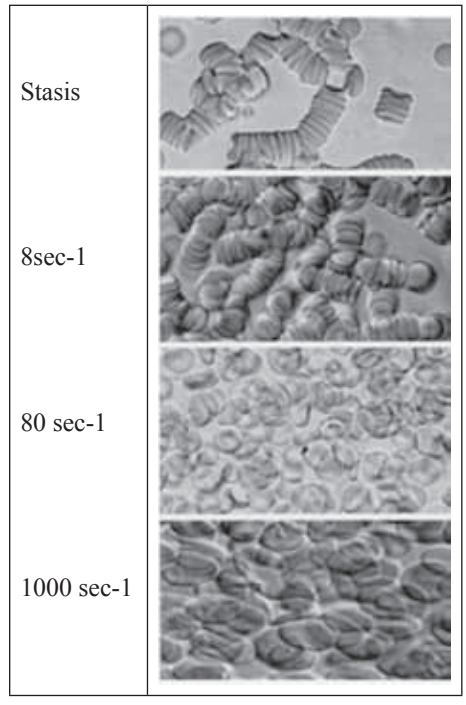


Fig. 2 – Apparent viscosity of blood with a constant hematocrit of 45% in relationship from different shear rates (From S. Chien [7])

properties of blood. It is a reversible dynamic phenomenon that can be observed *in vitro* and *in vivo* and is found to be responsible for much of the increase in viscosity at low shear rates. RBC aggregation is accepted to be responsible for the increased viscosity of blood samples measured by rotational viscometers[7,8]. In stationary flow, the apparent blood viscosity depends both on the hematocrit and on the shear rate applied[11]. The major role played by these parameters is illustrated in Figure 2 by Chien[7] by the variations of apparent viscosity or relative viscosity (Chien[8]). These results revealed that the high viscosity observed at low shear rates is due to reversible erythrocyte aggregation whereas the low viscosity observed at high shear rates is mainly correlated to red cell deformability[7, 8].

Erythrocyte aggregation is a complex dynamic process contributed by cellular and plasma factors [9-11,13,14,24,35]. However, the full mechanism of RBC aggregation is still unknown, at least rheologically and biomechanically[24,31,32,39-41]. Two hypotheses have been proposed for the non specific aggregation processes. The first one is based on the adsorption and cross-bridging properties of surface polymers. The interactions that occur between erythrocytes are characterised by their aggregation at low shear rates and by their dissociation at high shear rates. Therefore red blood cell aggregation occurs when aggregating forces overcome disaggregating ones. Factors essential to RBC aggregation are: local shear stress (Figure 3); the rheological properties of erythrocytes (cell count, deformability, surface charge, morphology); properties of macromolecules bridging adjacent

Fig. 3 – Shear rate dependence of the RBC aggregation



cells (molecular weight, conformation, charge); properties of the suspension medium (pH, temperature, osmolarity, ionic strength) and cell surface charge. The different types of interactions (physical or rheological) are due to the electrostatic repulsion. The second hypothesis is based on the concept of polymer exclusion or depletion between two RBC membranes; in other words, these surface molecules are not present between cells. It has been shown that hematocrit has a strong and nonlinear effect on erythrocyte aggregation.

Effect of RBC aggregation on in vivo blood flow

It is known that blood viscosity *in vivo* might be different from that measured *in vitro*. The effects of RBC aggregation on the flow properties of blood in microcirculation are much

more complex than a mere influence on blood viscosity. RBC aggregation enhances the phase separation of blood, with the formation of a cell-concentrated core and a greater plasma layer. As a result a severe RBC aggregation may cause discontinuous blood viscosity that will decrease as the plasma layer increases. Second, rheological effects on RBC aggregates depend on the geometry of the vessel under consideration. In large vessels, where rouleaux can rotate, RBC aggregation could lead to an increase in effective cell volume and in blood viscosity, whereas in smaller vessels whose diameters are only slightly larger than the RBC, rouleaux may line-up along the vessel axis during passage, thus minimizing effective viscosity and flow resistance[16].

Because RBC aggregation is a shear-dependent phenomenon, it is relevant to consider the shear conditions in various parts of the blood circulation network. The aggregation combined with yield stress is expected to reduce blood flow compared to that of non-aggregating system[24]. The influence of RBC aggregation on flow resistance is investigated in isolated and glutaraldehyde - fixed guinea pig hind limb[4]. The hind limbs of guinea pig are perfused using a constant flow pump attached to a catheter inserted in the abdominal aorta at various flow rates and pressure change at the entrance of the abdominal catheter is monitored. As shown in Figure 4 the flow resistance is significantly lower for RBC suspension contained Dextran 40, at flow rates below 0.42 ml/min.

There are proofs of a considerable increase of RBC aggregability in patients with arterial hypertension, ischemic stroke, etc. is an evidence

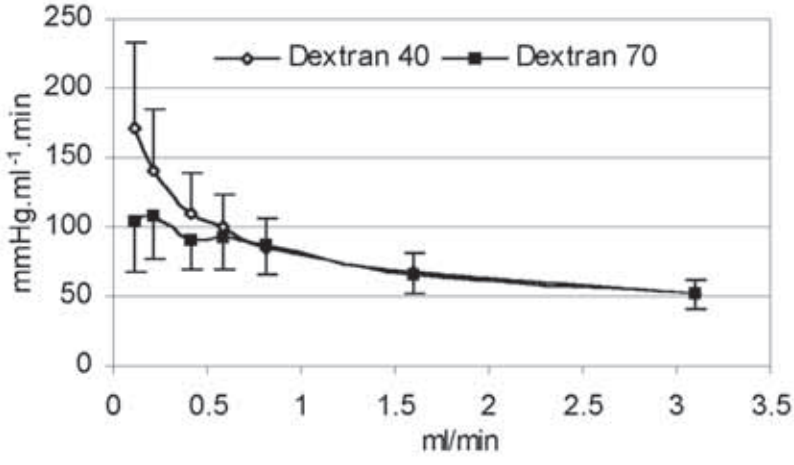


Fig. 4 – Flow resistance at various flow rates during perfusion with aggregating (Dextran 70) and non-aggregating (Dextran 40) red blood cell suspensions (From O. K. Baskurt [4])

of its key role in rising blood viscosity during development of these pathologies. The role of RBC intravascular aggregation in the disturbance of blood rheological properties in capillaries is analyzed[28]. The RBC aggregation is produced systematically (following intravascular administration of high molecular dextran) or locally (intensified exudation from individual capillaries) in rats' (intestinal mesenterium) and rabbits' (brain cortex) experiments.

The erythrocytes aggregation reduces blood flow compared to that of non-aggregating system what may result in local consequences as the increase in peripheral resistance, tendency to develop venous thrombosis or damage to tissues as a result of anoxia. Systemic increase of RBC aggregation leads to elevation of the arterial pressure. As to the local intensified aggregation, it results in a considerable slowing down of blood flow till full stasis in appropriate capillaries. In this latter case the systemic arterial pressure does not change and microvascular diameter does not decrease at any point all along the microvessels. This

furnishes ample evidence that intensified RBC aggregation is a factor that causes disturbances of blood rheological properties (by increasing the resistance to blood flow in the narrow microvessels) and represents itself an essential factor of blood rheological disorders in the microcirculation[27]. Comparison of the indices of hemorheological disorders in patients with cerebrovascular disorders with the same indices in the healthy control group demonstrated significant difference, especially sharp was increasing of RBC aggregability[26].

Measurement of erythrocyte aggregation and deformability in vitro

Erythrocyte aggregation and deformability play a vital role in microcirculation and hence their precise quantification is important to understand the rheology of blood in health and diseases. Techniques for measurement of deformability include micropipette aspiration technique, viscosimetric and rheoscopic measurement and filtration technique[12,15,16,18,19,44]. Micropipette technique, involves application of a specific negative pressure to allow the red cell to be aspirated through a micropipette of a known diameter. Deformability can be measured and also viewed through microscope. In viscomertric technique, correlating viscosity of the suspension with various shear rates leads to precise measurement of parameters involved in deformability. The problem with this technique is that at low shear rate, the aggregation interferes with the measurement. In rheoscopic method deformation of cells at various stresses is directly visualized through the microscope. The change in length and width of a stable oriented cell due to its elongation quantifies the deformability. Both these techniques require sophisticated systems and environment. The filtration technique is very advantageous over the others in terms of its simplicity. Here the erythrocyte suspension in physiological saline is allowed to flow through the torturous path of the microfilter under gravitation. The flow rate quantifies the deformability of erythrocyte. Figure 5 gives the block diagram of the optical hemorheometer used for the measurement of erythrocyte deformability[19]. It consists of syringe fixed into a filter holder in which the cellulose micropore (of 20 ± 5 μm) filter membrane (Scheilcher and Scull, Germany) is fitted. The filter holder is further connected into a three-way value, which is provided to control the flow. The optical arrangement for the measurement of flow rate includes an LED (640-nm) source and a detector placed on either side of the syringe. The scattered beam after passing through the observed volume is focused on the detector and the computer interfaced ADC card digitizes the analog output of the detector.

The purpose of the investigation of erythrocyte aggregation in steady flow as well as a ramp flow is to obtain information about the erythrocyte aggregability[1]. The comparison of the experimental data for a particular blood sample to normal blood reveals how much is the mechanism of aggregation affected by the pathological disturbances. On the other hand, the purpose of the measurement of erythrocyte aggregate forma-

tion in oscillatory flow is to determine the response of the aggregation mechanism to flow conditions simulating approximately blood flow in the vascular system[36].

For quantifying of RBC aggregation several techniques are available. They are based on the methods, classified as static and dynamic. Static methods can be connected with the studies on the sedimentation rate. Dynamic methods consist of studying on the reversible aggregation of red blood cells under blood flow conditions. The methods for quantification of RBC aggregation could be classified also as direct methods, based on microscopic observations of individual cells as they form aggregates and indirect methods, which measure microscopic rheological and other factors, which are affected by the cellular aggregation. Direct microscopic techniques, rheoscope, erythrocyte aggregometer, erythrocyte sedimentation rate test, low shear rate viscometers belong to these methods.

Different methods available to quantify erythrocyte aggregation include erythrocyte sedimentation rate[14] (ESR), viscometry, rheoscopy, microscopy and laser transmission and reflection technique.

The erythrocyte sedimentation rate (ESR) is one of the earliest methods for quantification of red cell aggregation. The ESR (measured in Westergen tubes) depends very much on the hematocrit of the sample, on the viscosity of plasma and on the temperature of the test. Viscosity exploits the property of increase in the apparent viscosity of the blood due to erythrocyte aggregation. However errors may be introduced due to

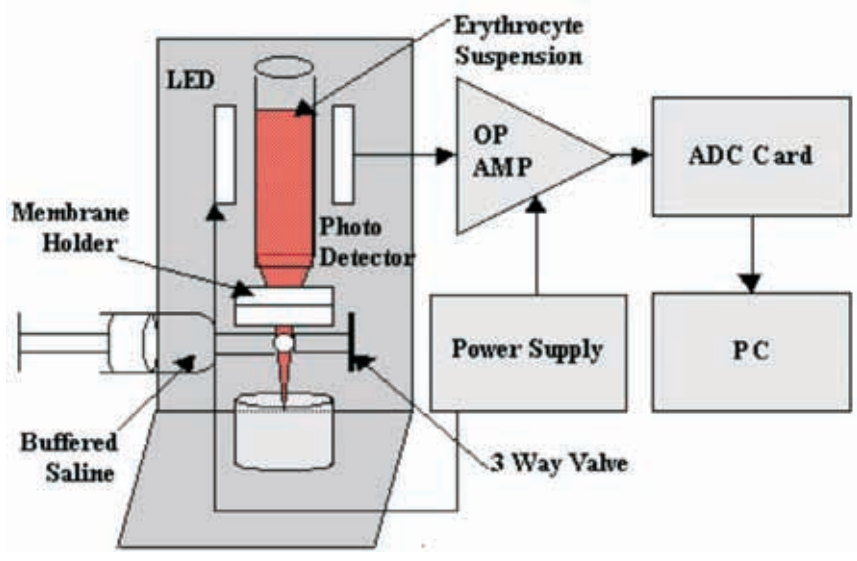


Fig. 5 – Block diagram of Optical Hemorheometer (From S. Jayavanth and M. Singh [19]).

changes in other factors on which viscosity depends. Some authors have developed viscometric systems to study the mean size of rouleaux according to the shear stress applied[2,13,18,31,35,41]. The viscometric methods belong to indirect methods and play important role in the wide variety of methods and instruments for assessment of RBC aggregation. The viscometric tests of RBC aggregation are efficient and relatively simple methods and every one of them gives a quantitative criterion for estimation of the observed phenomenon. S. Chien[7] (1981) proposed the viscometric aggregation index (VAI) for estimation of RBC aggregation. The relative viscosity of the red blood cell suspension at a fixed hematocrit of 45% in the autologous plasma η_{B45L}/η_P, is divided by the quotient of the viscosity of the RBC rigidity above the viscosity of the suspending medium.

M. Rampling *et al.*[34] (1986) analysed five methods for determination of RBC aggregation: microscopic ag-

gregation index, ESR, low shear couette viscometry, the Myrenne aggregometer and the Paar oscillating capillary rheometer. They showed that the low shear viscometry and the ESR were the most sensitive methods and had a linear response over a very wide range and that the low shear rate viscosity can be used as an index of RBC aggregation.

A viscometric method for determination of RBC aggregation is proposed by Ernst *et al.*[13] (1984). The resulting time dependent torque signal of the samples, submitted to a constant shear rate, reaches a peak, soon after starting the rotation and falls within seconds to a constant baseline, thereby describing an area "F" with the baseline. The shear history of the sample "F" is reproducible and shows a direct positive correlation with RBC aggregation as determined, for instance, in the aggregometer. J-F Stoltz *et al.*[41] (1984) compared three methods to approach rouleau formation: direct observation of rouleau formation (rheoscope); "erythrocyte aggregometer": with this technique the light, transmitted by the sample is integrated over a period of time and provide an index (Ia), laser reflectometer based on studying the light that is back-scattered by the blood, sheared in the gap of the viscometer. Their results showed that the indexes, measured by both reflection and transmission vary according to hematocrit and reach a maximum value at hematocrit levels of between 30 and 40%. They found significant linear correlation between the index Ia and the apparent viscosity at a given hematocrit value. The authors did not find significant correlation between the apparent viscosity and the reflectometer indexes, determined by the rheoscope.

The problem for correlation between the hemorheological measurement and the RBC aggregation was analysed by Resch *et al.*[35] (1992). They used four methods for aggregometer MA1 and Myrenne and measured "peak reading" of the disaggregating blood sample in the initial moment t_0 and 45 s after it in the moment t_1 by LS 30 Contraves viscometer. The measure for RBC aggregation is the difference between the apparent viscosity in the moment t_0 and t_1. The authors found a high correlation between the apparent viscosity and the RBC aggregation.

Many authors characterize the RBC aggregation by comparison the values of the viscosity $\eta_1=\eta\ (\gamma_1)$ for pathological blood with that of the control sample. Quemada[31] (1980) defines an index of aggregation as their relation at one and the same hematocrit, for example H=45% and at one and the same low shear rate:

$$A = \eta_1^{PAT}/\eta_1^{NORM}$$

Another approach for evaluation of aggregation is the rheoscopic method in which the aggregation level is found fairly correlated to the viscosity at different shear rates. Based on the intensity of the back scattered or transmitted light these methods provide an assessment of aggregation time and dissociation time and dissociation threshold (or a mean aggregation index).

This method yields quantitative measurement of rheological parameters such as viscosity, shear stress and shear rate, while aggregation as such is only estimated by visualiza-

tion. Microscopic technique is based on the method of direct observation of the aggregates. Microphotographs of aggregates are analyzed for number of aggregates per unit volume. In all the above methods, the information on the time course of aggregation is not available. Determination of time course of aggregation requires continuous recording of back-scattered or transmitted light signal through the suspension of erythrocytes under conditions where cells are allowed to sediment or flow under gravitational conditions. The computerised optical scattering technique – the Helium-Neon laser aggregometer uses this technique[19].

Figure 6 shows the block diagram of the Helium-Neon laser aggregometer[19]. The specimen chamber is made of optically flat glass with dimensions sufficient to allow required interaction between cells and sedimentation of formed aggregates. The transmitted intensity (TI) is detected by the photodiode. A computer interfaced ADC card digitizes the analog output of the photodiode. The card is programmed to acquire data through the entire process of aggregation at a desired sampling rate. The parameters, calculated from aggregation data as obtained by recording of on line TI are as follows: Aggregation size index (ASI): indicates the instantaneous change in the aggregate size given by the expression $N_{max} - N_{min}$. Aggregation sedimentation time index (ASTI): expresses the mobility of the aggregates in terms of sedimentation and it is measured as the time width at the base of a given fluctuation. The process completion time (PCT): Indicates the total time required for the completion of the sedimentation from the beginning till the TI reaches the intensity of I_0

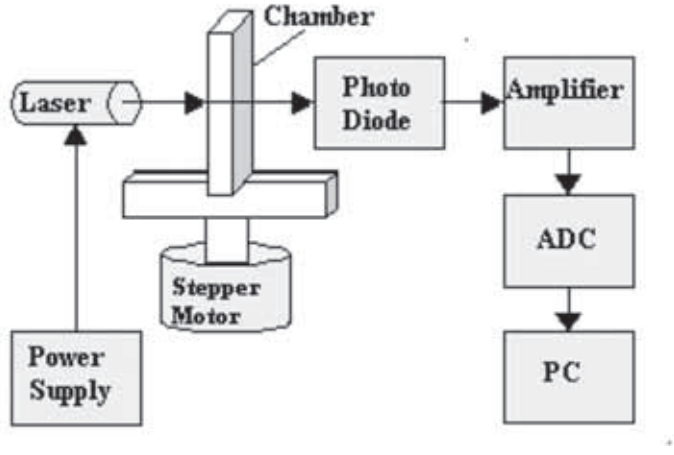


Fig. 6 – Block-diagram of He-Ne Laser Aggregometer (From S. Jayavanth and M. Singh [19]).

Among the various methods developed to study RBC aggregation, optical methods are the most suitable at the present time. Recently, optical aggregometry is the method of choice for many applications in clinical diagnostics[24,31,36,37,44]. The method is based on the time-varying detection of laser light scattering. Cells are hydrodynamically disaggregated by highly rotational shear flow for initialization and passed through a laser beam. The scattering signals respect to time, called syllectogram, give information about dynamic cell aggregation characteristics including aggregation indexes (*AI*), half-time ($t_{1/2}$) and *M*-index[36,37].

In such techniques, the variations of light transmitted or back-scattered by flowing blood cells are mainly due to red cell orientation and aggregation[43]. The principle of Fully Automatic erythrocyte aggregometer (Myrenne, GbH, Germany) is based on the transmission of infrared light through a blood suspension. The measuring principle of erythroaggregometer (Regulest, France) is based on

laser back scattering and the instrument analyzed the changes in back scattered light intensity when shear rates imposed on RBC suspensions are abruptly arrested. The measurement principle of Lazer assisted optical rotational analyzer (LORCA,RR Mechatronics, Hoorn, The Netherlands) is also based on measurement of back scattering laser intensity[17]. RBC suspensions in autologous plasma are sheared at 400 s^{-1} for 5 s for disaggregation and laser light reflection from the sample is recorded for 120 s, after a sudden stop. The obtained light reflection-time curve (i.e., sylectogram) is analyzed by a micro-computer and several parameters of RBC aggregation are calculated. The aggregation index "M" is equivalent to the "M index" of Myrenne aggregometer and corresponds to the area above the sylectogram. The aggregation index "A" is the ratio of this area to the sum of the areas under and above the sylectogram. The computer fits a double exponential equation to the sylectogram and calculates two time constants. The smaller time constant (tFast) has been used for comparisons in this study. Disaggregation shear rate (gTmin) was measured by shearing RBC suspensions at 11 separate levels of shear rate between 10 s^{-1} and 800 s^{-1}, and finding the shear rate at which the light reflection was maximum. Experimentally obtained parameters by these three optical methods have a stable and a good reproducibility.

Ultrasonic methods can be utilized *in vitro* and *in vivo* to obtain information on the aggregate structure of red blood cells. In vitro experiments on acoustical detection of erythrocyte aggregation of blood suspension, in flow or stasis, have been reported by many researchers[5,6,9,10,11,22,39]. Among the different methods proposed to study red blood cell aggregation, ultrasound provides unique opportunities as it can allow the analysis in a laboratory instrument and measurements in animals and humans. The characterization of RBC aggregation with ultrasound relies on the analysis of the signal intensity backscattered by the RBCs and their aggregates. Quantitative detection and measurement of the aggregation phenomenon (size of the existing red blood cell aggregates) can be achieved according to the experimental conditions and parameters used[5,6,9,10,11,22,39].

Ultrasonic techniques contribute and take more and more importance to characterize, both *in vitro* and *in vivo*, the structure and flow properties of blood or red blood cell (RBC) suspensions. Ultrasonic waves of frequencies 1-15 MHz easily propagate through soft biological tissues, thus providing qualitative and quantitative information on mechanical and flow properties of blood and red blood cell (RBC) suspensions. Two types of techniques allow investigating blood behaviours: echographic devices via amplitude detection and Doppler devices via frequency detection of the ultrasonic signal. Association of echographic and Doppler modes to investigate in routine simultaneously structure and velocity of blood is commercially available.

The ultrasonic backscattering coefficient, for example, depending on the dimension of the scattering centers encountered by ultrasonic waves informs on the size of red blood cell (RBC) aggregates present in the medium by amplitude analysis or on RBC aggregation kinetics

by frequency analysis of the echographic signal. All the echographic and Doppler methods are based on the reflection or scattering of ultrasonic waves by blood and red blood cell suspensions, at rest or in flow. Ultrasound scattering technique provides indeed a way to explore quantitatively the aggregation processes of red cells since the aggregates are usually much smaller than the ultrasound wavelength and, then, Rayleigh scattering theory can be used. Quantification of the ultrasonic backscattering coefficient from suspensions at rest or in controlled shear rate can be considered as an index of the mean size of aggregates[5]. The first example concerns *in vitro* ultrasonic characterization of aggregated RBC suspensions in shear flow and shows how the ultrasonic technique is able to quantitate physical and geometrical parameters of aggregates[6,39]. *In vitro* rheo-acoustical determinations were carried out to characterize the state aggregation of blood both on suspensions at rest and in controlled shear stress conditions[5,6].

A method, based on dielectric properties of dispersed systems was applied to investigate the kinetics of RBC aggregation and the formation and break-up of the aggregates[3,20,21,30]. Time variation of whole human blood conductivity σ and shear stresses under transient flow at rectangular and trapezium-shaped Couette viscometric flow were investigated under electric field of 2 kHz[3]. To investigate aggregation process in stasis and under flow conditions after subjected to shearing for 30 seconds to disperse all aggregates, RBC suspension was stopped or decreased to allow RBCs aggregation. Immediately after beginning and complete stoppage of shearing kinetics of conductivity and torque signals were recorded. If the higher shear rates had no further effect on σ values measured during shearing, the applied shear rate was considered to be sufficient high for complete dispersion of the aggregates.

CONCLUSION

The erythrocyte deformability, an important hemorheological parameter contributing to the exchange of metabolic products with the tissue environment, is attributed to the various constituents of the membrane and hemoglobin. Any variation of these may result in the impaired functioning of erythrocytes. Erythrocyte aggregation is a fundamental parameter of the rheological properties of blood and can be observed *in vitro* and *in vivo*. It is found to be responsible for much of the increase in viscosity at low shear rates as well as in many diseases.

The measurement of aggregation of erythrocytes by different methods and instruments is superior in terms of analysis of the kinetic process of the aggregation. The parameters obtained by the aggregation curve describe the dynamic nature of its mechanism. The deformability measurement by optical hemorheometer is simple. Computerised measurement of the filtration rate makes it precise. The parameters of aggregation and deformability obtained by the different methods and techniques serve as indices for comparison between healthy and subjects with diseases. Such a comparison may lead to a clue for diagnosis.

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