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Instrumental analysis applied to erythrocyte properties

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Abstract. The properties, structure and functions of the erythrocyte or red blood cell (RBC) have been known and evaluated by using simpler to more sophisticated technical devices. Microscopy, flow cytometry, spectrometry, spectroflurometry, aggregometer, ektacytometer zeta potential, amperometry, electrophoresis and centrifugation, are apparatus used to qualitatively and quantitatively characterize RBC, based on the principles of light transmission, light scattering spectroscopy, light absorption, fluorescence, light polarization, shear stress, shear rate, charge and molecular weight gradients. There is a symbiosis between the chemical, the physical and the mechanical principles of those instrumental analysis and the RBC properties to be measured. Here we briefly exemplify the relationship between the biochemical, biophysical and mechanical based techniques and the properties of the erythrocyte in healthy and disease.

Keywords: Erythrocyte, erythrocyte aggregation, erythrocyte deformability, erythrocyte exovesicles, nitric oxide

1. Erythrocyte bioavailability in oxygen and nitric oxide

Since the antiquity blood had fascinated the humans who associated it to curative power and magic process. In the seventeen century Jan Jacob Swammerdan (1637–1680) was the first scientist to observe erythrocytes or red blood cells (RBC) from frog blood under the light microscope. He was followed almost in same time by Antonje Van Leeuwenkoek (1633–1723) who performed the RBC observations also with light microscopy but equipped with lenses produced by himself (in http://micro.magnet.fsu.edu/primer/museum/swammerdam1670s.htm and http://pt.wikipedia.org/wiki/Anton_van_Leeuwenhoek). He observed that the erythrocyte can elongate up to three times their original dimension, can undergo aggregation and he estimated the diameter of RBC as 8.5 μm. The erythrocyte is a component of blood and its ability to delivery oxygen to all tissues of the vertebrates, makes it unique. The red color of the erythrocytes raises the curiosity in those scientists to look them through the light microscope. Hemoglobin, that is the carrier protein of oxygen from lungs to tissues, absorbs red and infrared light differently [1].

The percentage of haemoglobin saturated with oxygen can be measured in real time by pulse oximetry and is an indicator of the blood oxygen saturation [2]. Oximetry consist in a probe that can be attached to the patient‘s finger, nose, toe or earlobe lied to a computerized monitor which allows a visual waveform

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and an audible signal emitted with each pulse beat (the tone decreases with decreasing saturation). It is a non-invasive device to monitor the hemoglobin oxygen saturation (SaO2) of arterial capillary blood that uses a light source and a light detector. Desaturated haemoglobin absorbs red light and oxyhemoglobin infrared light. If the oximeter measures greatest absorbance in the red band it will indicate low saturation. If the higher absorbance is in the infrared band it means high saturation usually related with blood filled in the arteries. It needs attention to several interfering factors like exposition of patients to carbon monoxide or a situation of cardiac arrest [2]. For estimation of the tissue oxygenation status of patients it is necessary to quantify the partial arterial oxygen pressure (PaO2) and P50 assessments. The affinity of haemoglobin to oxygen can be described as the oxygen tension at which the haemoglobin is 50% saturated (P50). The value of P50 indicates the possibility of oxyhemoglobin deliver oxygen to tissue and can be evaluated mathematically applying the Hill equation developed by Lichtman et al. [3]. There is an instrument where the oxygen tension is detected by a Clark electrode and the oxyhemoglobin fraction (%HbO2) is evaluated by a dual-wavelength spectrophotometer and the dissociation curve (a relationship between the amount of oxygen dissolved in the blood and that is attached to the haemoglobin) is registered in a scanner [4]. High and low values of P50 indicate decreased and increased affinity of oxygen to haemoglobin respectively. The intra globular increased content of certain allosteric molecules, like 2,3 bisphosphoglycerate carbon dioxide, and the protons (decrease of the pH values) increased P50 value [5, 6]. Using circular dichroism spectroscopy large functional changes can take place, at molecular levels, between hemoglobin in R state (ligated with oxygen) and those heterotropic ligands that decrease the hemoglobin affinity for oxygen even in a full ligated state [7]. Changes in SaO2, PaO2 and P50 values were obtained when determined under physiological conditions like exposition to high altitude or during physical exercise [8]. At high altitude atmospheric pressure decreases, then PaO2 decreases and consequently low haemoglobin oxygenation occurs in non adapted healthy persons that start to manifest dyspnoea, tachycardia, tiredness or more complicated events. In adapted humans such as the highlanders those adverse issues are not found. They have higher values of P50 (30 mmHb versus normal 27 mmHg) [8].

During maximal exercise the amount of lactate increases, acidosis can occur in muscle, liver and kidney, blood flow can be compromised leading to a decreased tissue oxygen tension [8a]; the experimental measurements showed left shift in the oxyhemoglobin dissociation curve, lower P50 value (increase the haemoglobin affinity to oxygen) which reflect decreases delivery of oxygen [8].

Mutations on haemoglobin gene like in sickle cells present high values of P50 and lower PaO2 given by pulse oximetry [9]. Low haemoglobin affinity for oxygen and low oxygen saturation in peripheral blood was also verified by pulse oxymetry in patients presented mutation on α-globin chain, named haemoglobin Titusville [10].

It was found that the P50 was increased in hypertensive patients with diastolic pressure higher or equal to 130 mm Hg [11]. In a group of diabetic patients with different degrees of retinopathy normal levels of erythrocytic 2,3BPG and normal enzyme activities of the hexokinase, phosphofrutokinase and pyruvate kinase were obtained but in spite of these they presented high plasma glucose levels and decrease values of P50 [12]. This higher haemoglobin affinity for oxygen suggested transient decreases in RBC oxygen delivery with implications on retinal microvasculature structure [12]. At this time the authors proposed the influence of the glycosylated haemoglobin (HbA1c) concentration in the lower value of P50 as an explanation [12] which was not corroborated later [13–15]. A spectro-fotometric method was initially described [16] for quantification of HbA1c and was further modified by us [17]. Nowadays there are commercial diagnostic kits for quantification of glycosylated hemoglobin allowing diabetic patients to have it in their routine analysis. For clinicians the value of HbA1c serves as a marker of average blood glucose levels over the last 2-3 months.

It was shown that the P50 values are gender-independent and increased when adrenaline was added to blood samples obtained from healthy adult female and male [18]. In other "*in vitro*" studies after incubation of blood samples in presence of acetylcholine an increase of P50 values was verified demonstrating a decreased affinity of haemoglobin to oxygen [19]. An exogenous molecule like Spermine NONOate, a NO donor, was shown to increase the hemoglobin affinity for oxygen [19].

It must be noted that higher levels of hemoglobin oxygenation stimulate the anion exchange (band 3 protein) while deoxygenation accelerates the potassium-chloride co-transport and decrease the mechanical fluctuations of the membrane (CMFs) [20, 21]. The CMFs may be monitored by time-dependent light scattering from a small area of the cell surface by a method based on point dark field microscopy [21]. The mechanical fluctuations are associated with membrane displacement devoid to a contribution of hemoglobin, to thermal and metabolic energies and to erythrocyte deformability [21].

Acceleration of glycolysis (glycolytic enzymes leave the band 3 protein to cytosol), occurs concomitant with binding of deoxyhemoglobin to band 3 protein [22].

Beyond the ability of haemoglobin to deliver "*in vivo* oxygen" other factors related with the tissues ability to extract it and with microvascular haemodynamics are determinants for tissue oxygenation. The use at bed-side of sub-lingual device, like sidestream dark field imaging (SDF), allows the evaluation of blood flow characteristics [23]. SDF uses a polarized light to see and measure the number of small vessels either in occlusion or in perfusion [24]. For blood flow evaluation in the human tympanic membrane laser-Doppler flowmetry was used and ameliorated recently with a vibrometer approach [25, 26]. The identification of impaired microvascular blood flow by those noninvasive means can lead to early identification of patients at risk for peripheral or vascular and coronary artery disease [27, 28].

Nitric oxide was identified as a vasodilator in dependence of the intactness of the vascular endothelium in presence of acethylcholine [29]. Erythrocytes scavenge NO from endothelium bound to the heme of deoxygenate hemoglobin forming nitrosyl haemoglobin and tied to the cysteine of oxyhemoglobin beta chain originating S-nitrosohemoglobin [30, 31]. Electron paramagnetic resonance and photolysis-chemiluminescence techniques were used to quantify S-nitroso and nitrosylhemoglobin [30, 31]. Cristallographic analyses confirmed the binding of NO to the heme group of T state (deoxygenated) of hemoglobin while the NO binding to beta cysteine needs aerobic conditions [32].

The rate of NO influx into erythrocytes is greater when haemoglobin is oxygenated and in lower hematocrit than when is oxygenated [33]. The influx is blocked by Heinz body formation or by inhibitors of anion exchange also named band 3 protein [34].

When a situation of higher tissue oxygen demand exist a signal transduction mechanism occur involving cAMP with efflux, from erythrocyte, of adenosine triphosphaste (ATP) which is recognized by the purigenic receptor in endothelial cell and consequently NO synthesis is stimulated initiating vasodilation [35]. In situations of hyperemia the nitrosylhemoglobin values measured by electron paramagnetic resonance correlated with the tonometry data's of the endothelial function [36].

The visualization of nitric oxide inside erythrocytes was obtained with fluorescence microscopy digital images by loading with diaminofluoreceine-2diacetato (DAF-2D) that in presence of NO, oxygen and esterases originate triazolofluorescein [37]. The synthesis of NO with the participation of nitric oxide synthase (NOS) inside erythrocytes has been described using immunogold-labeling and electron microscopy imaging, immunofluorescence confocal microscopy, Western blotting, and reverse transcriptase – polymerase chain reaction [38]; RBC NOS present structural similarities with endothelial source [38]. Others used immunohystochemistry evaluated semi-quantitative immunoreactivity of the 1177 phosphorylated residue of NOS protein [39]. Other authors evidenced their doubts by gas

chromatography-mass spectrometry assays about the erythrocyte enzyme activity able to generate sufficient effective NO [40].

The NO effluxes from erythrocytes can me measured by an amperometric method [41]. NO diffuses through the gas-permeable membrane triple COAT of the sensor probe and is then oxidized at the working platinum electrode, resulting in an electric current. The redox current is proportional to the NO concentration outside the membrane and is continuously monitored with the electrochemical detection system and connected to a computer [41]. For more details see the review about the application of NO sensors im medicine [42]. A higher NO release from erythrocyte samples obtained from patients with diseases related with hypoxia and inflammatory states, namely sickle cell disease, hypercholesterolemic and hypertensive patients was verified, besides the fact that they sustain impaired erythrocyte deformability [43]. Glutathione is another important molecule that binds NO forming S-nitrosoglutatione (GSNO) which amounts inside the erythrocytes was quantified by a spectrophotometric method [44]. However using this method the efflux of GSNO from erythrocytes was only verified when erythrocytes were stimulated with acetylcholine and or timolol maleate [45].

2. Erythrocyte membrane properties and functions

The qualitative and quantitative observation of RBC membrane properties and functions highlight some of the blood flow behaviour in the vascular network. In the end of ninety century Ehrlich and Romanowsky used not successfully different aqueous dyes to stain blood smears and William Leisham and James Wright started to use methanol to fix the cells before staining to surpass that difficulty [46].

The shape of RBC can be observed in the optic microscope with limiting resolution of 0.2 microm and absence of the third dimension (thickness). The appearance of the scanning electron microscope strongly ameliorates those limitations allowing the visualization in 3D with tenfold of improvement in resolution [47]. Since the seventeen's electron microscope progress to eliminate the metal or carbon coating around the samples to be capable of "low vacuum" operation [48–50].

Besides its property to magnify cells it does not show the changing movements that characterize a living cell.

In 1953 the Nobel Prize in physics award Frederik Zemike for the development of the phase contrast microscope which was acquired by many basic science laboratory [51].

It continues facing the assessment of qualitative analyses without subcellular specificity that means do not distinguish below half the wavelength of light although the high magnification attained.

At same time the appearance of the fluorescence microscopy and of the confocal microscope brought new possibilities to study erythrocyte properties and binding to molecules. Now we are able to have molecular specificity and high spatial resolution but with loss of live cells in their native physiological state due the requirement of cell permeabilization and immunostaining [51a].

In October of 2014 the Nobel Price in Chemistry went to Eric Betzig, William Moerner and Stefan Hell for their development of a super-resolved fluorescence microscopy which brings optical microscopy into the nanodimension [52].

Using fluorescence confocal microscopy and flow cytometry it was possible to show that erythrocyte binds soluble fibrinogen in dependence of age in a way that the younger subpopulation of erythrocytes binds much more fibrinogen molecules than the older of the same blood sample [53]. All subpopulations, the older and the younger erythrocytes decrease its ability to bind fibrinogen when antibody CD47 was added previously. Noticed that CD47 is a membrane protein present in different cells with the purpose to

signalize them to be not "to eat" by phagocytes and consequently advertized notto be removed from blood circulation. Using zeta potential technique that measure the extension of interaction between two particles it was able to confirm the binding between erythrocyte CD47 and soluble fibrinogen in dependence of the age of RBC subpopulations [53]. For this study the erythrocytes were obtained from centrifugation of blood samples and are separated in subpopulations of different ages by percoll discontinuos gradient as described by Corsi et al. [54] and Venerando et al. [55] with some modifications [53].

Using phtalates esters mixtures of different gradients it is possible to isolate exovesicles produced from erythrocyte when submitted to fluorescent probes (usually used for membrane fluidity measurements) that were not visible in buffer suspensions [56]. In order to visualize them coloration for protein (Commassie blue staining), for acetylcholinestease (Ellman's reagent for the enzyme spectrophotometric assay) and for phospholipid content (phosphatidic acid coloration) were used [57, 58]. The importance of these coloration processes is to avoid the physically evaluation of exovesicles by using light scattering spectroscopy that is most expensive.

The human RBC in blood circulation has a prolonged life-span of 120 days and present susceptibility to aging and eryptosis (apoptosis) which produce exovesicles liberation [59, 60]. Acetylcholinesterase (AChE) is a biomarker of exovesicles originated from erythrocytes [56, 61] and also is considered as an index of RBC membrane integrity.

Increase enzyme activity of erythrocyte AChE has been reported in arterial hypertension, lateral sclerosis amyotrophic, and deficient in paroxysmal nocturnal hemoglobinuria [62–64]. Erythrocyte AChE is a very peculiar enzyme which is inhibited by high concentrations of acetylcholine (ACh) its natural substrate [65]. So, AChE originates active and inactive and less active complex forms of the enzyme according its association with the substrate (in concentration below Km) or with stronger inhibitors respectively [65].

The enzyme complex forms formed in the erythrocyte membrane between AChE and ACh or AChE and velnacrine (strong inhibitor) participate in the nitric oxide transduction mechanism associated with the band 3 protein phosphorylation/dephosphorylation through the Gi protein [66, 67]. In these studies, spectrophotometry, electophoresis and immunoblotting techniques were used and it was evidenced that AChE function and a chemical transducer [66, 67]. It was shown that the erythrocyte in the presence of acetylcholine increased its ability to delivery NO which disappears if fibrinogen was added [68]. Fibrinogen known as an acute phase protein maintains the NO inside the erythrocyte as shown in an *in vitro* study [69]. In hyperfibrinogenemia the scavenger ability of the erythrocyte to maintain NO inside prevails also in presence of the 4N1K the agonist of CD47 with increased GSNO, peroxinitrite, nitrite and nitrate concentrations [70]. Otherwise high fibrinogen levels concomitant with band 3 phosphorylation promote the efflux of NO from the RBC [71].

Phosphorylation of band 3 protein promotes the (i) displacement of the glycolytic enzymes to the cytoplasm from its N terminus and the approach of S –nitrosohemoglobin (ii) increase of the efflux of NO from erythrocyte, (iii) enzyme activity of AChE and (iv) the erythrocyte aggregation [22, 66, 67, 72, 73].

Erythrocyte aggregation is measured using either light transmittance or light back-scattering following abrupt stop of shear through or from red blood cell suspensions (anticoagulant blood or in autologous plasma) respectively [74]. Both measure the time course and the intensity of the aggregation but a faster time course was obtained with the light back-scattering if compared to the value resulting from the measurement done with light transmittance [74]. After shearing the blood sample the apparatus stop and the laser backscatter intensity from the samples versus time is recorded (syllectogram) before and after sudden stop (zero time). This method failed for blood samples taken from patients with very strong

RBC aggregates. The use of ultrasound with an echo probe substitute backscatter light and allow *in vivo* evaluations [75].

In the rotative cone-plate aggregometer, that disperses the blood cells by high shear stress (600/s) connected to a photometer, a syllectogram is also obtain (intensity of infra red light transmission versus time) that determines the extent of aggregation by integrating for 5 or 10 seconds [76, 77]. An update was performed connecting a computer to provide kinetic data of aggregation process [78]. Another apparatus was developed with shear rates from zero to 3500/s and measurements are based on changes in laser backscatter intensity [79].

Beyond those methods there are others based on (i) microscopy with computerized image analysis, (ii) low shear viscometry and electrical properties are also used and can be reviewed in the Handbook of Hemorheology and Hemodynamics [80].

Fibrinogen, lipoproteins, macroglobulins or immunoglobulins, plasma viscosity and hematocrit affect erythrocyte aggregation [81]. Plasma viscosity is an extrinsic influent factor in erythrocyte aggregation and if it is excluded by suspending RBC in standardized suspending media for example isotonic dextran solutions the greater or lower tendency to aggregate is called aggregability [82, 83]. The tendency of RBCs to reversibly aggregate and disaggregate influences blood flow [84].

RBC aggregation interfere with the measure of the erythrocyte sedimentation rate (ESR) assessed in routine clinical laboratories because it is recognized as a marker of acute phase of inflammation [85]. ESR is determined in vertically mounted tubes of defined length and diameter after one hour as first described by Westergren in 1921 [86]. Using blood samples with anticoagulant (citrate) the drop of RBC under the gravity force is also influenced by its size and shape as well the values of plasma viscosity, fibrinogen levels, hematocrit and room temperature where the test is done. Based on this method other was developed to spend only 20 min in the RBC drop by scanning twice the tube containing the anticoagulant blood sample with an optoelectronic light source [87]. A shorter assessment of ESR in 20 seconds was created using a microflow cell in a photometric rheoscope but the ESR values obtained were lower correlated with those assessed by the reference method of Westergren [88].

High erythrocyte aggregation values were shown in blood samples obtained from hypertensive patients besides the decreased values of plasma viscosity and hematocrit which evidenced the contribution of the intrinsic properties of RBCs on its tendency to aggregate [89–91]. The haemoglobin oxygen content did not cause interference in erythrocyte aggregation if measured by light transmittance [92].

Erythrocyte aggregation and plasma viscosity were elevated in patients with different degree of retinal vascular damage which are associated with plasma viscosity [93].

The association between the strength of large RBCs aggregates and the high plasma fibrinogen concentration was observed in patients with inflammatory bowel disease with detrimental microcirculatory blood flow in the intestinal microvasculature [94]. RBC aggregation was reported to be a biomarker of cardiovascular disease due to its association with other cardiovascular risk factors as observed in obesity, hypertension, dislipidemia and diabetes [95–98].

A review about the erythrocyte aggregation values obtained in patients monitorized by cross-sectional and longitudinal clinical studies was published [99].

Erythrocyte aggregation and deformability determine blood viscosity and microvascular perfusion [100, 101, 101a]. While erythrocyte deformability influences both blood flow in macro- and microcirculation, erythrocyte aggregation affected it predominantly in low-shear regions of microcirculation [102]. RBC when exposed to high shear stress and forced to pass through capillaries that can be as small as one quarter of the cell diameter are able to reversibly deform (change reversibly its form without alteration of the volume) which means present deformability [103].

The partially or totally aspirated erythrocyte into capillaries of 1 to 5 microns was quantified based on the amount of negative pressure yielding the shear elastic modulus of the membrane or the deformability [104]. It was a method not widespread due to its time consuming requiring especial skills. Several years have been passed to develop a microfluidic approach that provides morphological information at the single-cell level [105]. It was evidenced with this single-cell technique no changes in erythrocyte deformability when storage bank blood samples were assessed [105].

The first home technique to measure the ability of RBC to deform was developed by Reid et al. [106]. It is a filtration method based on gravity force or by application of a positive or negative pressure. The passage of a certain amount of erythrocyte suspensions (Ht 8%) through 3 to 5 microns pores was quantified by time taken in relation to buffer or by pressure-flow relationship [106]. Using the Reid et al. method in blood samples obtained from diabetic patients with microangiopathy a decreased filtration rate was verified [12]. Patients suffering from cerebral disorders presented also a decrease of the deformability index in a study which the Reid et al. apparatus [107]. Trying to simulate capillaries another device was constructed with flow channels connected to a microcomputer. This computer–controlled filtrometer measured the flow curve of a RBC suspension trough a filter membrane [108]. The apparatus contained U-shaped tubes and RBC suspension is place in one side and the filter is inserting at the collateral side of the U-shaped tube [108, 109].

A microchannel coupled with a microscope is named a rheoscope setup useful to visualize the RBC while being deformed instead to obtain mean of deformability index; it is available to follow shape changes under a specific shear stress [110–112].

Erythrocyte deformability can be determined using the Rheodyn SSD laser diffraction ektacytometer (or ellipsometry) that was initially developed by Goner et al. [113]. The Rheodyn SSD diffractometer determines erythrocyte deformability by simulating the shear stresses exerted by the blood flow and vascular walls on the erythrocytes. Erythrocytes are suspended in a high viscous medium and placed between a rotating optical disk and a stationary disk, where they are going to be subjected to well defined shear stress, which forces the erythrocytes to deform to ellipsoids and align with the fluid shear stress. A computer incorporated gives the analysis of the different ellipse, curves according the shear stress, and elongation index (EEI) are obtained respectively. The respective curve profile resulting from all EEI values in function of the shear stress applied can be linearized as a Lineweaver-Burk analysis procedure.

Beyond erythrocyte membrane composition and structural arrangement of its macromolecules, the internal viscosity and the ratio between RBC volume and its surface are important parameters on erythrocyte deformability [114].

Computer simulations compared with *in vitro* results were recently studied and show that the characteristic transient time depends on the imposed flow strength in RBC passing through small microcapillaries [115]. The numerical simulated data are validated by the theoretical analysis of experimental data which give the values of RBC 2D membrane viscosity and confirms the characteristic transient time value with those obtained by other technical devices [115].

Lower oxygenation of haemoglobin impaired the erythrocyte deformability [116]. PKC inhibitors also decreased RBC deformability [117]. It was evidenced that elimination of phosphorylation of protein 4.1 allows stronger interactions between membrane proteins with those of cytoskeleton [118].

Erythrocte deformability is improved *in vitro* by sodium nitropruside, an NO donor, as much as the deoxygenate state of haemoglobin [116]. Erythrocyte under shear stress by ektacytometer in presence of internal or external stimuli showed different erythrocyte deformability curves [119].

Carbenoxolona, an inhibitor of pannexin-1 hemichanel exporter of ATP, increased erythrocyte deformability in lower shear stress evaluated by ektacytometer [120].

Impaired erythrocyte deformability-dependence of plasma glucose level has been reported in diabetes with coronary artery disease that can increase morbidity in these patients [121]. Patients with nephropathy with or without diabetes presented decreased erythrocyte deformability assessed by ektacytometer [122].

Usually humans have their routine blood tests according their health system and physiological state. For that, clinical laboratory of analysis use more or less sophisticated Coulter counter instruments based on the detection of electrical conductance changes resulting from the passage of RBC in a conductive medium [123]. For example the RBC distribution width (RDW) is an important characteristic of the performance in athletes and present prognostic value in cardiovascular pathologies [124, 125].

An approach to measure simultaneously RBC area and volume under flow in microcapillaries, using high-speed video microscopy imaging was developed and data compared with Coulter counter and micropipette assays [126]. This technique overcomes the reliability of micropipette experiments, eliminates the conductivity of the suspension medium and assures the measurements of RBC surface area, (omitted in the routine Coulter count), volume, RDW and reproducibility of the results [127].

3. Conclusions

The oxygenation or deoxygenation status of hemoglobin depends on a myriad of properties inherent (i) to RBC (shape, membrane stability, metabolism, oxidative stress and hemoglobin structure and binding to nitric oxide and to allosteric modulators), (ii) to blood characteristics namely its composition, type of flow, rheology, (iii) to vascular environment related to hemodynamic properties, hemostatic processes, inflammation status, presence of atherosclerosis plaque and (iv) to tissues capacity and metabolic needs to extract oxygen.

The hemoglobin oxygenation level critically influences intracellular signaling pathways, action of hormones and or vasoactive agents, ion transport, and deformability of RBC.

The erythrocyte bioavailability in nitric oxide (NO) or its tendency to deliver or scavenge it depends on (i) endogenous and exogenous compounds and (ii) erythrocyte redox status and membrane protein phosphorylation degree; and affects erythrocyte deformability. All these erythrocyte properties and functions have been discovered among the years with the aid of instruments based on biochemical, biophysical and biomechanical characteristics of RBC. Computer simulations were also developed but were beyond the objective of the present review.

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