Biomechanical properties of red blood cells in health and disease towards microfluidics

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Red blood cells (RBCs) possess a unique capacity for undergoing cellular deformation to navigate across various human microcirculation vessels, enabling them to pass through capillaries that are smaller than their diameter and to carry out their role as gas carriers between blood and tissues. Since there is growing evidence that red blood cell deformability is impaired in some pathological conditions, measurement of RBC deformability has been the focus of numerous studies over the past decades. Nevertheless, reports on healthy and pathological RBCs are currently limited and, in many cases, are not expressed in terms of well-defined cell membrane parameters such as elasticity and viscosity. Hence, it is often difficult to integrate these results into the basic understanding of RBC behaviour, as well as into clinical applications. The aim of this review is to summarize currently available reports on RBC deformability and to highlight its association with various human diseases such as hereditary disorders (e.g., spherocytosis, elliptocytosis, ovalocytosis, and stomatocytosis), metabolic disorders (e.g., diabetes, hypercholesterolemia, obesity), adenosine triphosphate-induced membrane changes, oxidative stress, and paroxysmal nocturnal hemoglobinuria. Microfluidic techniques have been identified as the key to develop state-of-the-art dynamic experimental models for elucidating the significance of RBC membrane alterations in pathological conditions and the role that such alterations play in the microvasculature flow dynamics. © 2014 AIP Publishing LLC.

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I. INTRODUCTION

Red blood cells (RBCs) possess a unique capacity for undergoing cellular deformation to navigate across various human microcirculation vessels, enabling them to pass through capillaries that are smaller than their diameter and to carry out their role as gas carriers between blood and tissues.^{1–4} Pathological alterations in RBC deformability have been associated with various diseases⁵ such as malaria,^{6,7} sickle cell anemia,⁸ diabetes,⁹ hereditary disorders,¹⁰ myocardial infarction,¹¹ and paroxysmal nocturnal hemoglobinuria (PNH).¹² Because of its pathophysiological importance, measurement of RBC deformability has been the focus of numerous studies over the past decades.^{2,13–15}

Several comprehensive reviews have been published related to this issue,^{2,16–18} and the most recent have focused on the characterization of biomechanical properties of pathological RBCs, particularly involving sickle cell disease and *Plasmodium falciparum*-induced malaria.^{8,16,18–21} It is hence not the aim of this work to provide another detailed review of these pathologies but rather to highlight the need to provide further insight into specific pathological conditions. Reports on pathological RBCs are currently limited and, in most cases, not expressed in terms of well-defined cell membrane parameters such as elasticity and viscosity.

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Hence, it is often difficult to integrate these results into the basic understanding of RBC behaviour, as well as into clinical applications. Nevertheless, there is growing evidence that RBC deformability is a potential indicator of specific pathological conditions and may provide a label-free biomarker for determining cell status and properties of clinical relevance.

This review article will present data on RBC deformability in specific human diseases and is organized as follows. Section II discusses the rheological properties of healthy RBCs, which are the major determinants of cell deformability, whereas Sec. III presents a short reference to the most widely used experimental measuring methods. The next sections describe experimental studies on the deformation of single cells and/or cell populations for the following diseases: hereditary disorders (Sec. IV), metabolic disorders (Sec. V), adenosine triphosphate (ATP)-induced membrane changes (Sec. VI), oxidative stress (Sec. VII), and paroxysmal nocturnal hemoglobinuria (Sec. VIII). Sec. IX includes the concluding remarks and future research directions.

II. BIOMECHANICAL PROPERTIES OF HEALTHY RBCs

RBC deformability plays an essential role in the delivery of oxygen to tissues.⁵ Under physiological conditions, RBC deformability allows the flow of a single, biconcave disk-shaped RBC (Figure 1(A)), with dimensions of approximately $8\text{ }\mu\text{m}$ in diameter and $\sim 2\text{ }\mu\text{m}$ in thickness, through capillaries with diameters not more than $3\text{--}5\text{ }\mu\text{m}$, thus supplying oxygen to tissues of the body. As RBCs pass through the spleen, which acts as a highly effective filter, these cells are required to traverse extremely narrow endothelial slits with a diameter of $0.5\text{--}1.0\text{ }\mu\text{m}$. In the case of impaired RBC deformability due to pathological factors, splenic sequestration and destruction of RBCs are observed.^{2,13}

The deformability of an erythrocyte is attributed to three factors: (i) The large surface area-to-volume ratio of the biconcave disc, which presents a mean cell volume (MCV) of $\sim 90\text{ fl}$ and a mean surface area (MSA) of $\sim 135\text{ }\mu\text{m}^2$, which is significantly greater than the surface area ($97\text{ }\mu\text{m}^2$) of a sphere enclosing a volume of 90 fl ;²² (ii) the viscosity of the intracellular fluid (i.e., cytoplasmic viscosity), dominated by the presence of haemoglobin; (iii) the viscoelastic properties of the cell membrane, which is a composite material consisting of three layers, a carbohydrate-rich glycocalyx on the outer surface, a lipid bilayer capable of resisting bending, and an underlying protein network, which serves as the membrane skeleton that is responsible for its deformability, flexibility, and durability and aids in recovering the discoid shape.^{2,14,23} The RBC surface membrane presents a globular structure (Figure 1(B)) that reflects the skeletal integrity of the cell membrane. The latter, which is measured as surface roughness, is an indicator of the functional status of the cell,^{9,24} changing during a disease state or in the presence of environmental stimuli such as drugs.^{24–26}

FIG. 1. Scanning electron microscopy (SEM) micrographs of a typical healthy RBC (scale = $1\text{ }\mu\text{m}$) (A) and of the RBC membrane, showing globular structure (scale = 100 nm) (B). Adapted from Ref. 27.

The combination of the three factors listed above allows RBCs to undergo deformation, which facilitates their flow across the microvasculature. An abnormal increase in RBC rigidity has been associated with a change in any one or a combination of these factors.^{28–31} The mechanical behaviour of the RBC membrane is expressed in terms of three fundamental moduli: (a) the shear elastic modulus; (b) the area compressibility modulus; and (c) the bending modulus. The former is associated with a constant area of elongation or shear of the membrane. Area compressibility modulus, characterizing the resistance to area compression or expansion, is defined by the simple linear relation between isotropic membrane tension and relative area expansion $T = K \Delta A / \Delta A_0$,^{2,32} and corresponds to the surface dilation without either shear or bending. Bending modulus, representing the curvature of RBC membrane, is important in driving rest shape changes, without either shear or expansion.^{33,34}

When transient conditions occur, such as in relaxation experiments to model vascular bifurcations and changes in vessels cross section, it is possible to measure the RBC relaxation time constant, defined as the time that a deformed RBC takes to recover its original biconcave shape. It is given by the ratio of the elastic shear modulus and the membrane surface viscosity, which represents the delay of shape change due to internal friction.^{35–37}

Another elastic modulus often used in the atomic force microscopy (AFM) measurements^{38,39} is Young's modulus, which is the measure of the stiffness of an elastic isotropic material and is defined as the ratio between the stress along a certain axis and the strain along the same axis.⁴⁰

The experimental methods used so far to measure the membrane moduli and the other RBC properties are described in Sec. III.

III. TECHNIQUES FOR MEASURING THE BIOMECHANICAL PROPERTIES OF RBCs

Several methods have been developed to measure the membrane properties of RBCs; however, most investigators are continuously challenged with the need to develop models that simulate blood circulation in physiological conditions. The available experimental methods can be divided into two categories: instruments that measure whole blood or diluted RBC suspensions, and single-cell techniques.

The former, which include the rotational viscometer⁴¹ and ektacytometer,^{31,42,43} are considered as high-throughput methods; however, these do not take into account heterogeneity or size differences within the sample population of cells. Rotational viscometers have been used in determining RBC cytoplasmic viscosity by studying red cell suspensions in fluids of different viscosities such as high-molecular weight dextran;⁴¹ however, it does not provide information on geometry and membrane properties of RBCs. A rheoscope, which consists of a counter-rotating cone and plate rheometer that allows microscopic observation of blood samples placed in a flow chamber, is suitable for the study of the microrheology of blood cells and for estimating the relaxation time constant of RBCs.^{44–46} In this microrheologic approach, density-separated cells are subjected to graded levels of shear, which measures the steady-state elongation and the time of shape recovery after an abrupt cessation of the imposed shear. In ektacytometry, a laser diffraction viscometer, RBCs' deformability is measured as a function of suspending medium osmolality. The deformability index (DI) curves contain information on RBC surface area, surface/volume ratio and internal viscosity. DI (often called Elongation index, EI) is defined as the ratio $(L - W)/(L + W)$, where L and W are respectively the major and minor axes of an ellipse that represents a RBC.^{47,48} The curve for normal blood samples reaches a maximum at 290 mOsm/kg, indicating that normal RBCs have an optimal deformability when exposed at the tonicity to which they are normally exposed. The DI max represents an equilibrium value between the surface/volume ratio and the intracellular viscosity. When the suspending medium osmolality decreases, DI decreases too, reaching a minimum at about 135 mOsm/kg. DI min indicates the maximum possible volume that a cell can reach before hemolysis. Pathological blood samples are characterized by differences in the shape of the profile DI vs osmolality as compared to the normal one. These kinds of instruments do not provide a direct

measure of the viscoelastic constants, although the output of some parameters could be related to RBC deformability.

Single-cell experimental methods include micropipette aspiration,^{36,49–51} optical tweezers,^{52–58} flickering analysis,^{59–63} AFM,⁶⁴ and, recently, microfluidics^{4,65,66} and ultrasounds.⁶⁷ Micropipette aspiration (Figure 2(A)) was one of the first techniques used to measure either RBC geometric (surface area and volume) or viscoelastic (surface viscosity, relaxation time constant, and shear, bending and area compressibility moduli) properties. These measurements are affected by the dependence on the size of the pipette used in the experiments (reliable comparisons can only be made between samples measured with the same pipette) and, in general, on the conditions of the experiments. Flickering analysis of RBC membrane is based on the large fluctuations of the cell shape, and is correlated to RBC mechanical properties, such as bending modulus, membrane tension and cytosolic viscosity. A recent study combines RBC membrane flickering with micro-Raman analysis, with the aim to study how oxygenation and hydration state can affect RBC mechanics.⁵⁹ RBC geometric parameters have also been studied using the hanging cells method (Figure 2(B)), where single RBCs⁶⁸ or blood droplets²² are hanging off the edge of the underside of a glass coverslip. Optical tweezers (Figure 2(E)), optical trapping, and other microscope-based techniques have been widely used to determine RBC membrane properties. AFM, a powerful technique for high-resolution imaging of any surface including those of cells, allows the characterization of the mechanical, electrical, and magnetic properties of cells,^{69,70} as well as the measurement of cell deformability in terms of the Young's modulus (Figure 2(D))³⁹ and of membrane surface roughness.^{9,25,26} In addition, full-field laser interferometry,⁷¹ a non-invasive optical technique, has been used to quantify the material properties of living cells at the nanometer and millisecond scales. In particular, this

FIG. 2. Overview of methods for characterizing the biomechanical properties of RBCs. (A) Aspiration of an RBC into a micropipette.⁵¹ (B) Topography of a healthy RBC by full-field laser interferometry. The scale bar is 2 μm, and the colorbar scales are in μm. Adapted from Ref. 71. (C) Three-dimensional (3D) topographical image of an RBC as measured by AFM.³⁹ (D) The distribution of the magnitude of the acoustic velocity around a levitated cell (undeformed). The acoustic velocity magnitude is in m/s. Adapted from Ref. 67. (E) Optical tweezers stretch an RBC that was loaded using a force ranging from 0 to 340 pN. From left to right: experimental observations, computed contour maps of the constant maximum principal strain distribution and one half of the full 3D biconcave shape (simulations with the cytosol). Adapted from Ref. 58. (F) Microcapillary flow (diameter = 10 μm): RBC transient shape at start-up using the same experimental conditions. Flow is from left to right. Adapted from Ref. 35.

technique has been used to retrieve the RBC membrane properties by quantifying RBC membrane fluctuations^{71,72} (Figure 2(B)).

Earlier, single cell-based methods allow the estimation of membrane properties that affect RBC deformability; however, these are also associated with specific disadvantages such as being low-throughput and static techniques. The stress imposed on the cells during analysis may not fully represent the actual conditions occurring during microcirculation, thus making it more difficult to evaluate the results in terms of clinical relevance. An alternative way to apply mechanical stresses to deform cells is based on ultrasonic radiation force that acts directly to the cell membrane. The cell can be osmotically swelled before the experiments, in order to obtain a spherical shape, that makes small deformations easier to detect⁶⁷ (Figure 2(D)).

Recently, the development of microcapillary flow techniques (Figure 2(F)) and microfluidic devices (Figure 3) have successfully circumvented the problems associated with single-cell characterizations, combining high-throughput analysis and physiologically relevant flow fields.^{4,73–75} Microfluidics originated from an experimental program that was performed in the '90s by the Defense Advanced Research Projects Agency (DARPA) of the US Department of Defense, with the aim of developing microsystems that could be used as detectors for chemical and biological threats.⁷⁶

Microfluidic devices, coupled with automated image analysis, are suitable for point-of-care applications,⁷⁷ allowing to test a large number of cells by using microcirculation-mimicking patterns,⁷⁸ transparent substrata such as polydimethylsiloxane and glass, and video microscopy.

Examples include visualizations of cell deformation as a function of pressure drop, in which the classical parachute-like shape observed *in vivo* was observed in *in vitro* experiments as well,^{66,79–84} estimations of cell membrane viscoelastic properties such as RBC shear elastic modulus and surface viscosity by using diverging channels,⁶⁵ measurements of the RBC time recovery constant in start-up experiments,³⁵ cell characterization by electric impedance micro-flow cytometry,⁸⁵ and single-cell microchamber array (SiCMA) technology^{86,87} (Figures 3(D1) and 3(D2)). The latter applies a dielectrophoretic force to deform RBCs and used image analysis to analyse RBCs shape changes, allowing the evaluation the deformability of single RBCs in terms of Elongation Index %, defined as $(x - y)/(x + y) \times 100$, where x and y are RBC major and minor axes, respectively. Dielectrophoretic force has been also used for the real-time separation of blood cells for a droplets of whole blood.⁸⁸

FIG. 3. Microfluidic experimental studies of RBC flow using microcirculation-mimicking models. (A) RBC membrane elastic modulus and surface viscosity are measured by using a converging-diverging geometry. Adapted from Ref. ⁶⁵ and Reproduced by permission of The Royal Society of Chemistry. (B1) Low- and (B2) high-magnification images of deformed erythrocytes in glass microchannels. Adapted from Ref. ⁸³. (C) Flowing of RBCs in channels of different widths.⁸⁹ (D1) Schematic diagram of a chamber unit for RBC deformability measurement (SiCMA chip), and (D2) Fabricated SiCMA chip with an enlarged view of the chamber array.⁸⁶

Recently, RBC geometrical parameters such as RBC volume, surface area, and distribution width (RDW), which are a measurement of the size variation as well as an index of the heterogeneity that can be used as a significant diagnostic and prognostic tool in cardiovascular and thrombotic disorders,⁹⁰ have been measured in microcapillary flow using high-speed microscopy.^{81,91,92}

The use of different techniques leads to various measured values, meaning that deformation of RBCs deeply depend on the deformation protocol. This fact has been widely discussed in recent papers which state that the mechanical response of RBC is not linear.^{93,94} The wide discrepancies resulting from the use of different techniques can be observed in the large standard deviation of the values presented in Table I, where the average values of the geometric and mechanical properties of healthy RBCs present in the literature are reported together with their related experimental techniques.

In order to identify which technique has been used to measure the RBCs biomechanical properties, in Figure 4, eight categories have been reported, such as micropipette, flickering, viscometry, microcapillary flow/microfluidics, ektacytometry, AFM, optical tweezers, and other, where the voice “other” includes reflection interference contrast micrograph, microscopic holography, hanging cells, flow channel, magnetic field, laminar flow system, and optical interferometric technique. Data from both healthy and pathological RBCs (Hereditary membrane disorders, metabolic disorders and ATP-induced membrane changes, oxidative stress, PNH, Malaria, and Sickle cell anemia) have been considered to realize Figure 4.

IV. HEREDITARY MEMBRANE DISORDERS

Hereditary disorders involving the erythrocyte membrane include spherocytosis (HS), elliptocytosis (HE), ovalocytosis, and stomatocytosis (HSt). These syndromes are caused by a deficiency or dysfunction of one of the membrane skeletal proteins and constitute an important group of inherited haemolytic anemias.^{10,117–119} HS and HE are characterized by the presence of spherical-shaped erythrocytes and elliptical, cigar-shaped erythrocytes, respectively, and are the most common disorders of RBC membranes, affecting 1 in 2000 people of North American and Northern European countries, at a prevalence of 1 in 5000.¹¹⁷ Southeast Asian ovalocytosis (SAO), which is characterized by rounded elliptic or ovaloid red cells, is an uncommon variant of HE that occurs in some parts of Southeast Asia. All these diseases result from mutations in genes that code for different erythrocyte membrane proteins such as spectrin (alpha and beta), ankyrin, glycoporphin C, band 3 protein, and proteins 4.1 and 4.2.^{21,118,120–122} RBC membrane properties in hereditary disorders have been mostly evaluated by ektacytometry,^{47,123–125} which does not

TABLE I. Geometric and mechanical properties of RBCs.

	Healthy RBC	Techniques
Volume (μm^3)	89.4 ± 17.6	Micropipette, ^{95–98} hanging cells, ^{22,68} flow channel, ⁹⁹ microscopic holography, ¹⁰⁰ microcapillary flow ⁸¹
Surface area (μm^2)	113.8 ± 27.6	Micropipette, ^{95–98} hanging cells, ^{22,68} flow channel, ⁹⁹ microscopic holography, ¹⁰⁰ microcapillary flow ⁸¹
Cytoplasmic viscosity (mPa/s)	6.07 ± 3.8	Rotational viscometer, ^{41,101} cylindrical micropores, ¹⁰¹ magnetic field ¹⁰²
Surface viscosity ($\mu\text{N s/m}$)	0.7 ± 0.2	Micropipette, ^{32,36,103–105} ektacytometry, ³⁷ microfluidic device ⁶⁵
Shear elastic modulus ($\mu\text{N/m}$)	5.5 ± 3.3	Micropipette, ^{49,103–106} optical tweezers, ^{52,107} ektacytometry ³⁷
Bending elastic modulus ($\times 10^{-19}$ Nm)	1.15 ± 0.9	Micropipette, ^{33,108} reflection interference contrast micrograph, ^{109,110} AFM, ¹¹¹ flickering analysis, ^{59,108,112} optical trapping, ¹¹³ optical tweezers ¹¹⁴
Relaxation time constant (s)	0.17 ± 0.08	Optical tweezers, ⁵² micropipette, ^{36,103,105} optical trapping, ¹¹³ rheoscope, ^{45,115} microcapillary flow, ³⁵ microfluidic device ⁶⁵
Area compressibility modulus (mN/m)	399 ± 110	Micropipette ^{32,104,116}
Young's modulus (KPa)	26 ± 7	AFM ³⁹

	Micropipette	Flickering	Viscometer	Microfluidics	Ektacytometry	AFM	Optical tweezer	Other
Volume	✓			✓				✓
Surface area	✓			✓				✓
Cytoplasmic viscosity		✓	✓					✓
Surface viscosity	✓			✓	✓			
Shear elastic modulus	✓			✓	✓		✓	✓
Bending modulus	✓	✓				✓	✓	✓
Relaxation time constant	✓		✓	✓		✓	✓	
Area compressibility modulus	✓							
Young's modulus						✓		
Deformation Index			✓		✓			

FIG. 4. Techniques used to measure a specific RBC biomechanical property.

provide direct measurements and is limited to descriptions of shifts in the DI curve vs osmolality relative to the control. Ektacytometry results (Figure 5 and Table II) suggest that hereditary disorders (HS,^{10,126} HE,¹⁰ SAO,^{10,127} and HST¹⁰) cause a decrease in the deformability of the RBC membrane, shown by the shift in the pathological DI vs Osm curve relative to that of the control.

Decreasing in surface area are characterized by a decrease of DI max and by a general shifting of the curve in the direction of the increasing osmolality, in particular, DI min is found at higher osmolality values (Figure 5(A)). However, decreased values of DI max not always indicate reduced surface area. An increased membrane shear modulus tends to reduce the maximum DI, and, in general, a slight shift of the whole curve to lower osmolality is present (Figures 5(B) and 5(C)). Cynober *et al.*⁴⁷ described the changes in membrane surface area, surface area-to-volume ratio, and cell volume of RBCs based on the osmotic profile in both non-splenectomized and splenectomized patients with HS; their study detected a decrease in

FIG. 5. Microscopy imaging of RBCs (top panel) and ektacytometry curves, in which grey and black lines pertain to control and patient cells, respectively, for: (A) Hereditary spherocytosis. Black arrows indicate the typical spherocytic shape, red arrows indicate acanthocytes (RBCs with spiked membranes), and blue arrows indicate basophilic red cell due to the anemic state; (B) elliptocytic (red arrows) and ovalocytic red cells (blue arrows); (C) stomatocytic red cells (black arrows). Adapted from Ref. 10.

TABLE II. Mechanical properties of RBCs affected by hereditary spherocytosis, elliptocytosis, ovalocytosis, and stomatocytosis.

	Control	HS	HE	SAO	HSt	Techniques
Shear elastic modulus ($\mu\text{N/m}$)	5.4 ± 3.4	5.04 ± 1.32	6.5 ± 2.1	...	...	Micropipette ^{129–131}
Relaxation time constant (s)	0.19 ± 0.086	0.086 ± 0.06	0.04 ± 0.02	...	...	Micropipette ^{129–131}
DI _{max}	0.45 ± 0.05	0.3 ± 0.27	0.65 ± 0.2	0.3 ± 0.05	0.51	Ektacytometry ^{10,47,125–127,132}
Young's modulus (KPa)	26 ± 7	43 ± 21	...	...	...	AFM ³⁹

membrane surface area in all of the studied patients and suggested that the percentage of microcytes may serve as a good indicator of disease severity.

The spectrin-dependent behaviour of the DI has also been studied,¹²⁸ which showed that DI_{max} (maximum osmotic profile as a function of osmolality) is associated with the spectrin content of cells, and that DI_{max} decreases with the severity of spectrin deficiency. Waugh^{129–131} obtained similar results using micropipette experiments and suggested that spectrin deficiency is one of the causes of membrane abnormalities. Spectrin is regarded as the main energy-storing molecule of the RBC membrane and is responsible for elastic shear deformation. The shear modulus of the RBC membrane thus largely depends on the surface density of spectrin in the membrane. In the same studies, membrane shear modulus and viscoelastic time recovery constant were evaluated for HS and HE.

Membrane shear modulus was slightly smaller for HS and HE cells compared to the control, whereas viscoelastic recovery time constant values were similar to that of the control (Table II). These two parameters are available for HS and HE only, because SAO and HSt are very rare pathologies, and thus, it is more difficult to find donors.

In the past few years, AFM³⁹ has been used in studies involving the mechanical properties of erythrocytes from patients with HS in terms of Young's modulus. This parameter was higher in HS erythrocytes compared to normal cells, indicating possible changes in the organization of the RBC cytoskeleton.

Currently, osmotic gradient ektacytometry is the best diagnostic reference technique for hereditary disorders. Flow cytometry has also been used but can often generate false positive results, which are often circumvented by microscopy analysis of cellular morphology. Thus, because microscopy analysis is considered as the most important step in the diagnosis of hereditary disorders, a novel system such as a microfluidic device that combines RBC image analysis and the quantification of membrane properties such as the three fundamental membrane moduli (the shear elastic modulus, the area expansion modulus, and the bending modulus) and the relaxation time constant could be a novel tool in the diagnosis of hereditary disorders and could help to establish the relationship between membrane defects and the clinical status of the patient. The initial steps have been conducted towards this goal through the application of a microfluidic-based method, the SICMA^{86,87} technology, that is able to measure the Elongation Index of individual cells in a heterogeneous population, allowing the recognition of subpopulations of pathological cells by the comparison with the healthy ones. A 30% reduction in RBC deformability has been observed in spherocytosis by using SICMA technology.⁸⁶

V. METABOLIC DISORDERS

A metabolic disorder occurs when abnormal chemical reactions disrupt normal metabolism, which is the process that converts food to energy at the cellular level. Metabolic diseases affect the ability of a cell to perform essential biochemical reactions involving the transport and/or processing of proteins, carbohydrates, or lipids. The most common metabolic diseases include diabetes, hypercholesterolemia, and obesity, which are recognized as multiplex risk factors for cardiovascular disease.¹³³

A. Diabetes

Type II diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and results from either low insulin levels or insulin resistance. It is the most frequent cause of renal failure and legal blindness, and one of the major risk factors of cardiovascular diseases.^{134,135} Several studies have shown that diabetes can be associated with enhanced plasma and whole blood viscosity and abnormal RBC membrane architecture, in which the diabetic state can cause a reduced level of cell deformability, as well as complications such as microvascular disease and microangiopathy.^{136–139}

It has been observed that RBC cytoplasmic viscosity largely depends on the physicochemical properties of haemoglobin¹⁴⁰ such as glycosylation, whose increase is due to diabetic-related hyperglycaemia. Thus, altered membrane lipid-protein interactions and increased glycosylation could alter RBC membrane viscoelastic properties that contribute to diabetic progression.^{141–143} Abnormalities in the RBC membrane in diabetic patients involve an elongated shape with membrane folding around spontaneously formed fibrin fibres (Figures 6(A)) and a smooth membrane (Figure 6(B)).⁹

In the 1970s and 1980s, filtration^{144–148} and micropipette¹³⁹ experiments showed that diabetic RBCs present an impaired deformability,¹⁴⁹ and that these take more time to recover their discoidal shape. On the other hand, rheoscope analysis¹¹⁵ did not detect any differences between control and diseased cells in terms of the time required for shape recovery.

La Celle¹⁵⁰ and Sewchand *et al.*¹⁵¹ also did not observe any abnormalities in diabetic RBC deformability. More reliable and systematic measurements on RBC deformability were performed by Tsukada *et al.*⁸³ by using a simple glass microfluidic device as a model for microvessels (Figures 3(B1) and 3(B2)). The classical RBC parachute shape in flow was characterized in terms of a DI (Deformability Index), which is defined as the ratio between cell length and width. The RBC DI of diabetic patients was smaller than that of healthy donors, thus indicating an impaired deformability. Recently, Brown *et al.*¹⁵² used a filtration technique to describe the DI as the rate of filtration of a dilute suspension of RBCs through membranes with straight channels under a constant negative pressure, compared to the rate of filtration of an equal volume of buffer. Their study detected impairments in RBC deformability in diabetic patients. In the last decade, a disposable ektacytometer^{153,154} equipped with a laser diffraction system and slit rheometer has been developed to measure RBC deformability in terms of DI (referred to as EI in Refs. 153 and 154). Also, in this case, an impaired RBC deformability was observed in diabetic patients compared to non-diabetic controls.

Despite the incidence of diabetes and the extent of impairment in RBC membrane properties that may affect blood flow in microcirculation and tissue oxygen supply in vascular diabetic complications, only a few reports have described quantitative measurements of RBC deformability in diabetes.

One of the most important consequences of impaired RBC deformability is the impaired perfusion at the tissue level, which is a complication of diabetes mellitus.^{155,156} The development of high-throughput techniques to obtain quantitative data on viscoelastic properties of

FIG. 6. SEM images of diabetic RBCs: (A) RBC showing lengthened ultrastructure; (C) RBC showing smooth membrane (Scale = 1 μ m). Adapted from Ref. 9.

RBC membranes could be important to delineate the correlation of impaired RBC deformability with the complications of diabetes, such as microvascular disease, microangiopathy, and nephropathy, which are not well understood at the moment. Moreover, such high-throughput techniques could be used to test the effect of drugs for reduce whole blood viscosity at the micro-circulation level, since it is significantly enhanced in diabetes.¹³⁵

B. Hypercholesterolemia

Hypercholesterolemia is a metabolic disorder characterized by high levels of cholesterol in the blood, which is transported in the plasma as lipoproteins. Low-density lipoprotein (LDL)-cholesterol is recognized as a risk factor of atherosclerosis, hypertension, and coronary heart disease.^{157–159} The direct effects of cholesterol on blood flow include growth of atherosclerotic plaques that reduce the lumen of coronary arteries, as well as blunted endothelium-dependent vasodilation at the coronary microcirculation level that leads to an impairment of myocardial perfusion. Indirect effects of hypercholesterolemia involve blood rheology; in fact, a high level of cholesterol may affect whole blood viscosity, platelet activation, and RBC deformability, leading again to impaired coronary circulation.¹⁶⁰ By applying the laser diffractometric method,^{160,161} the responses of deformed RBCs to shear stress at increased flow speeds have been measured in hypercholesterolemic patients, which have revealed a decrease in RBC deformability. Unfortunately, this work, as well as filtration-based studies,¹¹² did not provide a direct measure of the viscoelastic constants, but the output was instead based on some parameters that were related to RBC deformability. On the other hand, micropipette experiments¹⁶² provided measurements of the intrinsic viscoelastic properties of both cholesterol-enriched and cholesterol-depleted RBCs. The former showed a flat, pancake-shaped RBC, as previously observed by Cooper *et al.*,¹⁶³ with an irregular contour (Figure 7) and a diameter bigger than that of the controls. The latter showed a smaller diameter than the control cells, and some cells presented a stomatocyte-like shape.

Thus, by using this technique, any significant alteration in the shear elastic modulus and surface viscosity has been considered as a consequence of the changes in the membrane cholesterol content. EI¹⁶⁴ as measured by using a Rheodyn (described in Sec. III) has been measured in patients with familial hypercholesterolemia. The results showed that patients had lower EI values than the controls. Recently, Forsyth *et al.*¹⁶⁵ utilized a microfluidic flow-focusing device to examine the relationship between cholesterol and ATP release from RBCs and showed that changes in membrane cholesterol can cause alterations in RBC deformability and, thereby, disruptions in ATP release. Because of the limited and inconsistent reports regarding hypercholesterolemic RBC membrane properties, further work is needed to establish the significance of RBC membrane alterations in this disease, as well as on blood flow at the coronary microcirculation level.

C. Obesity

Obesity is a widespread metabolic disorder associated with several cardiovascular risk factors such as diabetes, arterial hypertension, and hypercholesterolemia. Because of the presence of different degrees of obesity and diverse methodologies, contradictory results have been

FIG. 7. SEM images of cholesterol-enriched RBCs. Adapted from Ref. 163.

published regarding RBC deformability in obese patients.^{166–169} Some studies describe an increase in membrane cholesterol/phospholipid ratio, whereas only one study involved patients with morbid obesity.^{170–173} A recent study focused on the evaluation of RBC deformability in a group of patients with severe and morbid obesity without other cardiovascular risk factors.¹⁷⁴ RBC DI was measured using laser diffractometric techniques in terms of the EI, although it seems that RBC deformability does not decrease in obese patients without other concomitant cardiovascular risk factors. Thus, for obesity, as well as for hypercholesterolemia, there is a need to further investigate the correlation between obesity and alterations in the RBC membrane.

VI. ATP-INDUCED MEMBRANE CHANGES

Several studies^{175–177} have shown that the shape of RBCs is influenced by metabolic activities that regulate the intracellular concentration of ATP (adenosine triphosphate, an energy supplying molecule)¹⁷⁸ based on the fact that some sites of the RBC skeleton, such as the spectrin network, may absorb chemical energy from its contact with ATP.¹⁶ The mechanistic links involved in ATP release induced by RBC deformation have been also recently reviewed.¹⁷⁹ Modifications in RBC shape could be related to changes in ATP levels, and different experimental techniques have been proposed to clarify the relationship between RBC flow dynamics and ATP.^{104,114,165,178,180,181} Filtration experiments were conducted to determine the effects of ATP depletion on membrane deformability, which showed that the RBC membrane properties were influenced by the metabolic state of the cell, which in turn is regulated by ATP.¹⁸⁰ Simulation of a hemispherical deformation of the RBC membrane using a 3- μm micropipette has shown that ATP-depleted cells have a decreased level of deformability. The ATP-depleted cells may be unable to pass through narrow capillaries in the microcirculation and spleen, which contain 3- μm pores. By microscopic observation, an increase in RBC rigidity has been observed in ATP-depleted blood, suggesting the key role of ATP in the maintenance of RBC membrane properties.¹⁸⁰ Micropipette experiments provided data on the shear elastic modulus, the area compressibility modulus, and the surface shear viscosity for ATP-depleted RBC membranes;¹⁰⁴ however, no significant differences between fresh and ATP-depleted RBCs have been observed. No dependence on ATP has been also observed by measuring the change in RBC membrane fluctuations combining microscopy and image processing.⁶³

Recently, optical tweezers¹¹⁴ have been used to study the membrane-cytoskeleton interactions in terms of RBC membrane fluctuations; an increase in the RBC membrane bending modulus has been identified as the mechanism responsible for the reduction in membrane fluctuations. However, few years later, fluctuations analysis and optical traps⁹⁴ gave opposite results. Quantitative evidence obtained using full-field laser interferometry techniques⁷¹ shows that ATP facilitates the dynamic fluctuations in the RBC membrane. One of the factors influencing the structure and the mechanical integrity of the spectrin network and the overall shape of the RBC is the remodelling of the coupled membranes powered by ATP.^{71,72}

Despite the number of works present in the literature, the dependence of RBC deformation on ATP is still controversial. This is probably due to the complex cell mechanics and, consequently, to the cell deformation protocol. Indeed, although information on the mechanical properties of RBCs in relation to ATP are essential in understanding specific medical conditions such as hypertension and efficacy of blood transfusions, a physiologically relevant regime of deformation is not completely understood.

VII. OXIDATIVE STRESS

Under physiological conditions, RBCs are able to defend against the continuous exposure to oxidative stress, by the intervention of antioxidant molecules that inactivate reactive oxygen molecules and enzymes that convert toxic compounds in forms that can be excreted by cells.¹⁸² However, abnormal response of cells to oxidative stress can cause toxic effects through the production of peroxides and free radicals, leading to damages in RBC skeleton, in normal mechanisms of cellular signaling, in membrane fluidity, and, consequently, it can affect the passage

TABLE III. Mechanical properties of PNH erythrocytes.

	Control	PNH erythrocytes	Techniques
Relaxation time constant (s)	0.19 ± 0.086	0.26 ± 0.07	Micropipette ¹²
Shear elastic modulus ($\mu\text{N/m}$)	5.4 ± 3.4	6.3 ± 1.43	Micropipette ¹²
Surface viscosity ($\mu\text{N}\cdot\text{s/m}$)	0.71 ± 0.23	1.58 ± 0.54	Micropipette ¹²

through the microcirculatory network. Thus, impaired RBCs deformability caused by the damages to the structural components of the membrane worsen some pathological situations related to high oxidative stress, such as diabetes, and to microcirculation, such as heart failure and myocardial infarction.^{183,184}

Several investigators measured impaired RBC deformability in presence of oxidative stress by using techniques such as ektacytometry.^{185–190} To overcome the classical limitations related to ektacytometry measurements (i.e., poor accuracy and quantification of the amount of rigid cells in the sample), studies on single cell response to oxidative stress have been carried out in the last years. By the analysis of the membrane thermal fluctuation spectrum, mechanical properties of RBC, such as bending and elastic moduli, have been evaluated, reporting changes in the elastic constant of RBCs exposed to oxidative stress *in vitro*.¹⁹¹

More recently, a microfluidic approach has been proposed, demonstrating the presence of RBCs damaged by oxidative stress in terms of cortical tension,¹⁹² with the aim to discriminate between good stored and bad stored RBCs for transfusion, an important issue of clinical interest. Thus, although this approach does not give information about the relevant membrane parameters, such as bending and elastic moduli, relaxation time constant, and surface viscosity, it provides a cost-effective, high throughput, and reproducible method for measuring RBC deformability in presence of oxidative stress, and opens the way for further microfluidic studies.

VIII. PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

PNH is a rare (1–2 new cases per million per year and affects less than 200 000 people in the US population, according to the Office of Rare Diseases of the National Institutes of Health) acquired clonal disorder caused by a mutation in the X-linked phosphatidylinositol glycan class A (*PIG-A*) gene that leads to absence of protective proteins on the RBC membrane.¹⁹³ PNH is characterized by complement-mediated haemolysis, venous thrombosis, and bone marrow suppression.^{194,195} PNH erythrocytes are quite vulnerable to complement-mediated lysis because of a reduction or complete absence of two complement regulators, CD55 and CD59, both of which are GPI-anchored.^{195,196} Despite the importance of membrane viscoelastic properties in the survival of RBCs,^{129,180,197} only one study, to my knowledge, has examined the RBC membrane properties in PNH. Smith¹² utilized micropipette techniques in measuring the elastic modulus, viscosity, and time constant of RBCs affected by PNH. The mechanical properties of abnormal RBCs are impaired compared to normal cells, as shown in Table III.

IX. CONCLUDING REMARKS

In this article, mechanical properties of both healthy and diseased RBCs have been reviewed, focusing on the role of impaired RBC deformability in various pathologies such as hereditary disorders (spherocytosis, elliptocytosis, ovalocytosis, stomatocytosis), metabolic disorders (diabetes, hypercholesterolemia, obesity), ATP-induced membrane changes, and Paroxysmal nocturnal hemoglobinuria. *In vitro* experimental models that are commonly used to measure RBC deformability have also been described. RBC membrane properties in hereditary disorders have been mostly measured by ektacytometry,^{47,123–125} a laser diffraction-based method that does not provide direct measurements and is limited to describing membrane properties in terms of shifts in the DI curve *vs* osmolality from the control. Thus, based on these measures, an impaired RBC deformability has been characterized. The case is different with

metabolic disorders because reports describing this association are limited and traditional measurement techniques have been used in the few published reports on this matter, which may have contributed to the generation of conflicting results. In some cases, as for example, for PNH, only one study¹² has examined the RBC membrane properties, finding an impaired cell deformability.

The role of RBC deformability has thus been established in only a few disorders.

One major disadvantage of the experimental methods used for the evaluation of RBC biomechanical properties is that the stress imposed on the cells is far from the one actually experienced during in microcirculation. This fact may distort the results, which in turn may decrease the clinical relevance of the study. Although enormous progress has been made, our understanding of the role of RBCs deformability in such diseases is still far from complete. Despite the number of complications generated from a decrease in RBC deformability such as impaired blood flow in vascular complications of the diseases, particularly at the microcirculation level, a quantitative knowledge of RBC membrane properties is needed.

Therefore, it is necessary to develop state-of-the-art dynamic experimental models for elucidating the significance of RBC membrane alterations in pathological conditions and the role that such alterations play in the microvasculature flow dynamics by using well controlled and physiologically relevant mechanical environments. Since many traditional biochemical and biological assays such as flow cytometry,^{198–201} DNA extraction,^{202,203} and polymerase chain reaction,^{203,204} have already been transferred onto integrated microfluidic devices to provide multi-parameter information to target cells, it is possible to develop a novel high-throughput system, based on microfluidics techniques, for the measurement of RBC membrane properties. Such a system should allow high-speed/high-magnification observations of the flow of RBCs in model systems that well represent the human microvasculature. Recent reports have focused on the healthy cell⁶⁵ and on a few pathologies, i.e., malaria^{205–207} and sickle cell anaemia.^{208,209} On the other hand, data are limited related to the biomechanical properties of RBCs in common pathologies such as hereditary and metabolic disorders that involve severe complications in blood circulation at the microvasculature level due to impaired RBCs deformability. Thus, additional experimental studies are needed to extensively examine the mechanical properties of RBCs in various diseases to establish the correlation between RBC mechanical properties and patients clinical status.

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