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REVIEW ARTICLE

The Role of Cytoskeleton of a Red Blood Cell in Its Deformability

Shivangi Paradkar and Priya Gambhire[*]

Abstract | The red blood cell (RBC) is unique in terms of its structure and function when compared to other cells in the blood and body. Its anucleated characteristic and biconcave shape (indicative of high surface area to volume ratio) render it deformable. This deformability is useful during circulation when the red blood cell has to traverse capillaries smaller than its size. The cytoskeleton of the red blood cell, a two dimensional sheet like structure with dynamic linkages, plays a major role in its deformability. The interdependent relationship between the cytoskeleton and RBC deformability under various conditions such as metabolism, hematologic and systemic disorders and senescence is reviewed.

Keywords: *Red blood cells, Deformability, Spectrin*

1 Introduction

Biological systems are interesting subjects for mechanical studies owing to their unique structural properties. Being one of the earliest studied biological systems, historically, blood and specifically the red blood cell (RBC), enjoys special attention due to its mechanical properties.[1] The RBC surface is soft enough to undergo thermal fluctuations yet the cells are able to sustain high shearing, repeatedly, within the circulation.

Deformability is the change in morphology (compared to its original shape) that a cell can undergo, reversibly. It is the most important mechanical property of the RBC, originating in its structure and responsible for its efficient functioning. RBCs are produced within the bone marrow through the process of erythropoiesis. During this, the progenitor cells lose their nucleus and achieve a biconcave discoid shape to form mature RBCs. This process results in the RBC having an excess surface area for the contained volume, a property which the cell uses to assume energy minimizing shapes while traversing through narrow capillaries.

Being the most abundant of the cells present in blood, RBCs impart blood its viscosity. Hence, in order to maintain homeostatic blood flow, RBCs need to have the right amount of deformability which is periodically tested in the spleen. The endothelial slits in the spleen act as mechanical filters barring passage back to circulation to the least deformable cells. The cell deformability, therefore, becomes a factor in deciding when the cell is to be removed from circulation. As they age RBCs are removed from circulation within the spleen. This takes place between the 110 and 130th day of their life cycle.[2]

As the RBC deformability forms an important parameter in all its functions, its origin, and factors affecting it, are of significant importance. Studies have shown that the RBC cytoskeleton contributes significantly to its deformability. The focus of this review is to present the literature on RBC cytoskeleton limiting itself to the understanding of its structure, viscoelastic properties, factors it affects and is affected by. This discussion is presented in the forthcoming sections.

2 Structure of Red Blood Cell Membrane

The red blood cell membrane is composed of lipids and proteins. A schematic of the membrane arrangement is provided in the Fig. 1. The lipids exist as a bilayer with asymmetric distribution within the inner and outer leaflets.[3] The proteins exist either as peripheral or integral proteins. The peripheral proteins in turn are classified as cytoskeletal and anchoring proteins based on their function. The cytoskeletal proteins exist as a two dimensional scaffold unlike the transcellular actomyosin cytoskeleton found in most

[1] *Centre for Biosystems Science and Engineering, Indian Institute of Science, Bangalore, Karnataka 560012, India.*
**gpriya@iisc.ac.in*

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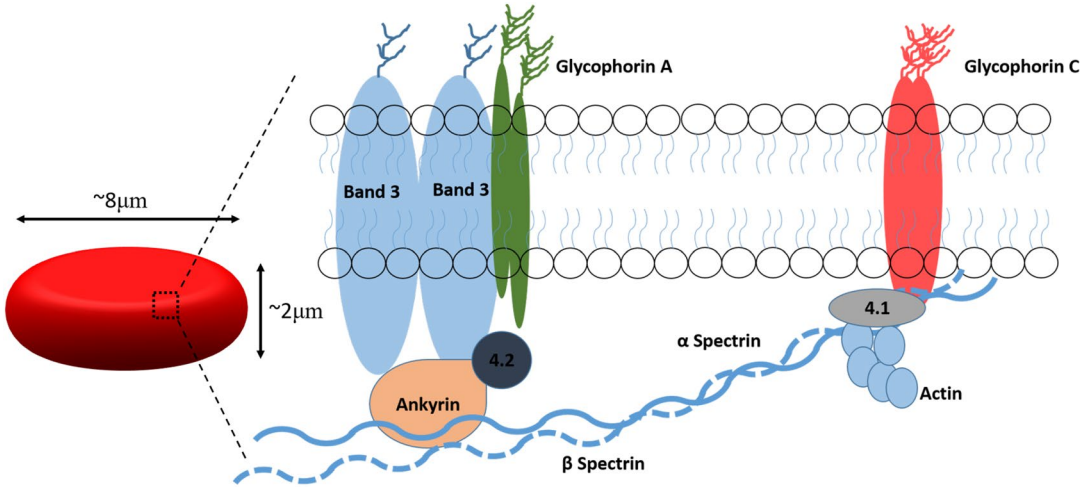

Figure 1: A schematic representing the red blood cell cytoskeleton arrangement.

eukaryotic cells. They form junctions with the integral proteins which exist within the lipid bilayer via the anchoring proteins.[4,5]

Extensive protein analysis studies have helped in determining the composition of the RBC cytoskeleton[6–9] Band 1 and 2 are high molecular weight protein molecules renamed α and β spectrin respectively. Other lower molecular weight proteins found are the bands 3, 4.1, 4.2, 4.5 and 4.9. Apart from spectrin, the cytoskeleton includes band 5 and 2.1 which are actin and ankyrin, respectively.

With the advent of techniques such as freeze fracture- and immuno- electron microscopy it was discovered that spectrin, most abundant of the cytoskeletal proteins, exists as a heterodimer where α and β are its subunits.[10,11] These heterodimers then form hexagonal tetramers which constitute the cytoskeletal network.[12] Spectrin alongwith actin and protein 4.1 forms a junctional complex[13,14] which connects to the glycophorins within the lipid membrane while spectrin alongwith ankyrin and protein 4.2 forms another junctional complex which connects to the band 3 integral protein.[15]

3 Red Blood Cell Deformability

3.1 Structural Basis

An RBC has to go through repeated mechanical constrictions throughout its life-cycle. Its membrane structure imparts it the ability to deform and recover. The red blood cell membrane has a viscoelastic nature. The lipid bilayer with the transmembrane proteins behaves as a viscous fluid while the cytoskeletal protein layer shows a solid-like elastic behavior.[16] Perturbing the skeletal-skeletal protein and skeletal-anchoring protein interactions is shown to negatively affect the RBC deformability and stability indicating the role of cytoskeleton in its deformability.[17] To understand how the molecular design of cytoskeleton translates to macroscale viscoelasticity multiple studies have focused on the dynamic changes in the structure of the cytoskeletal and membrane proteins. The heterodimers of spectrin in their relaxed state exist as loosely coiled double helix and only bind on ends to form tetramers. The lengths of the heterodimers and tetramers are approximately 97 nm and 194 nm, respectively.[18] In native state, the tetramers are found to assume a length of approximately 70 nm and have a very low energy of 2.5 RT (where R is the universal gas constant and T is the temperature) for doubling their length to a maximum of ~200 nm upon extension.[19–22] At the molecular level, the spectrin molecule has repeats which are found to be dynamic imparting the protein with flexibility.[23] Based on this structural information, the cytoskeleton is modeled to behave as a linearly extending elastic spring where certain spectrin molecules extend while others compress effectively maintaining constant surface area.[17]

3.2 Constitutive Models

On the basis of this structural information, constitutive models for RBC mechanics are developed. These models help better understand RBC deformation behaviour during flow. The bilayer which consists of lipids held together with hydrophobic forces resists area dilatation and bending

but flows easily upon shearing while the spectrin cytoskeleton is resistant to shear forces. A mathematical description of the red blood cell should, therefore, consider the moduli of area compressibility, bending rigidity and shear elasticity.

Along these lines, one of the many approaches to model the complex elastic behavior of the RBC is using the neo-Hookean or Mooney–Rivlin model. Pozrikidis[24] use it to model RBC deformation during capillary flow. More commonly, the Skalak law[25] is used to model RBC behaviour as it intrinsically considers the contribution from area dilatation. It was used by Kellar and Skalak[26] to obtain novel RBC dynamics seen in flow experiments such as tumbling and tank-treading. To obtain the shear rate dependency of these deformation regimes, Abkarian et al.[27] used the Kelvin-Voigt viscoelastic model to describe RBC mechanics.

Continuum based numerical RBC simulations also make use of one of the above constitutive models to describe cell mechanics which when combined with methods to simulate the fluid flow have been successful in capturing large deformation behavior seen in experiments.[28,29] Another approach to model RBC behavior are the particle based models where techniques such as dissipative particle dynamics are used. A comprehensive review of numerical RBC models can be found in Li et al.[30]

3.3 Measurement Techniques

All the above models make use of values of RBC elastic moduli to predict accurate deformation behavior. Various methods have been used to measure these properties, the most common being: micropipette aspiration, optical tweezers, atomic force microscopy, ektacytometry and microfluidic approaches.

In the micropipette aspiration technique, RBC is held within a pipette tip via suction pressure. This is manipulated in a controlled manner aspirating the cell further into the tube. The aspiration pressure and dimensions of the deformed RBC are used to obtain the mechanical parameters. For single RBCs micropipette aspiration is quite popular as it can be used to obtain most of the elastic moduli. Typical values of moduli for normal RBCs, obtained through this technique are $\kappa = 7\mu$ N/m , $\kappa_B = 1.8 \times 10^{-19}$ N/m and $K_A = 193$ dynes/cm, where κ, κ_B, K_A are the shear, bending and area compressibility modulus, respectively.[31–33]

Another approach to obtain RBC mechanical properties is by optical tweezing. Focused laser beams are used to trap silica beads (placed diametrically opposite on the RBC surface) and move them stretching the cell.[34] The optical tweezer approach provides the modulii of elasticity as the micropipette aspiration technique. The atomic force microscope, on the other hand, is used to obtain the Young's modulus of the cell. In this technique, a microtip attached to a cantilever is used to locally indent the RBC and its retraction force is measured. Typical Young's modulus for a normal RBC is reported to be approximately 26 ± 7 kPa and changes drastically for diseased cells.[35]

In contrast to the single cell measurement methods discussed above, microfluidic and ektacytometry methods are more suitable for studying RBC populations. In ektacytometry RBCs are sheared in a rheoscope and diffraction patterns of the deformed RBCs obtained from an incident laser beam are used to estimate a deformability index.[36] This technique is used in commercial instruments to measure RBC deformability and is being replicated using microfluidic techniques to make point of care devices.[37,38]

4 Factors Affecting RBC Cytoskeleton

RBC deformability enables it to pass through narrow capillaries ensuring perfusion to every tissue within the body. But the intricate RBC cytoskeleton, responsible for its deformability is affected by several factors throughout the cell's four month life span.

4.1 RBC Metabolism

RBCs lack nuclear material but carry all the essential enzymes required for metabolism. During their lifetime they do produce a limited amount of ATP via glycolysis. The RBC ATP, among many other functions, is used for both static (maintaining biconcave shape) and dynamic (fluctuations, deformation) changes within the cytoskeletal proteins. The biconcave resting shape of the RBC is shown to be energy dependent as depleting ATP from the cell over time resulted in them assuming a smooth spherical shape.[39] In addition to its role in maintaining the resting biconcave shape of RBCs, ATP is seen to contribute to the thermal fluctuations seen in the membrane bilayer. This active component to the membrane fluctuations is seen to have a root-mean-square (r.m.s) length comparable to the distance between the cytoskeletal junctional complexes. Hence, leading to the conclusion that ATP dependent formation and breaking of junctional complexes contributes to undulations on the RBC surface.[40] ATP

depletion is seen to have a reversible decrease in RBC deformability via an increase in membrane calcium.[41] Stiffness of the membrane is also found to increase which is attributed to reduction in spectrin phosphorylation.[42] It is also reported that upon deformation, RBCs release ATP to regulate the vascular tone[43,44] indicating an intrinsic relationship between RBC metabolism and its deformability.

4.2 RBCs in Disease

4.2.1 Disorders of Membrane Proteins

The cytoskeleton regulates RBC deformability through folding and unfolding of spectrin and dynamic linkages of anchoring proteins. Any disruption to these elements lead to disorders accompanied with chronic anemia. Hereditary spherocytosis (HS) and hereditary ovalocytosis (HO) occur as result of decrease and increase in the cytoskeleton and lipid bilayer linkages, respectively. Both these disorders cause a loss in cell deformability but the cell stability is not affected to a great extent in HO. HS RBCs, on the other hand, show a loss in membrane due to the ineffective linkage to cytoskeleton, resulting in cell shape being close to spherical.[45]

Disorders in the cytoskeletal proteins themselves such as deficiency in protein 4.1, disturbance in spectrin dimer-tetramer equilibrium can lead to disorders such as hereditary elliptocytosis (HE, wherein cells acquire an elliptical shape) and hereditary pyropoikilocytosis (HPP, wherein cell have microcysts on the surface). RBCs in all these disorders exhibit loss of deformability and are cleared within the spleen leading to hemolytic anemia and splenomegaly.

4.2.2 Disorders of Cytoplasmic Protein

Sickle cell Disease (SCD) is an inheritable blood disorder caused by a single point mutation in one of the hemoglobin (Hb) gene. This mutation triggers the polymerization of the deoxygenated Hb (HbS) and leads to the formation of long rod like stiff fibers which compel the RBCs to assume an elongated (sickle) shape (sRBCs).[46,47] Patients with SCD suffer from chronic anemia due to increased hemolysis, vaso-occlusive crises and in certain cases tissue/organ damage.[48] SRBCs are stiffer than normal RBCs.[49,50] Presence of HbS leads to an increase in cytoplasmic viscosity contributing to alteration of rheology of sickle RBCs[51] Altered rheology of sickle cells is also attributable to the increased association of HbS with the membrane proteins (such band 3 protein).[52–54] Studies show that sRBCs and HbS are more prone to generation of reactive oxygen species (ROS) and auto-oxidation, respectively.[55,56] Increased ROS production leads to loss in membrane deformability as hemoglobin, cytoskeletal and transmembrane proteins are oxidized.[57,58] Additional contributions to cytoskeletal changes in SCD are due to phosphorylation of cytoskeletal membrane proteins[59]and increased concentration of calcium ions in the membrane.[60]

4.2.3 Infectious Diseases—Malaria

Infectious diseases such as malaria directly affect RBCs. The parasite, *plasmodium falciparum* enters the host RBC (trophozoite stage) and multiplies to form daughter cells (schizont stage). The infected RBC (iRBC) eventually ruptures and releases the daughter parasites (merozoite stage) which continue infecting other cells. During the trophozoite stage, iRBC membrane forms nanoscale knob-like protrusions.[61,62] The knob proteins form additional linkages with the spectrin-actin-protein 4.1 junction increasing the cytoskeleton-bilayer adherence. [63] Cytoskeletal proteins such as adducin, tropomyosin and Rac1 which are part of the spectrin-actin junction are reduced. At longer times post infection, large holes appear in the cytoskeleton and key proteins such as spectrin and ankyrin are proteolysed either via the RBC's calpain-1 or parasitic proteases.[64] The length of the spectrin filaments is found to increase to almost 74% of its original value with the progression of the infection.[62] At the schizont stage of infection, iRBCs are found to have increased adhering properties to endothelium. This helps the iRBCs escape clearance by the immune system and increases morbidity due to vaso-occlusion.[65]

4.2.4 Chronic Diseases—Diabetes Mellitus

Non-hematological diseases also affect RBCs. Most prevalent of these is the metabolic disorder of diabetes mellitus (DM) which leads to a systemic increase in plasma glucose concentration. RBCs in diabetic patients (dRBCs) are found to have a larger than average size, exhibit lower concavity and a decrease in surface roughness.[66] One reason for the increase in cell volume observed in the case of dRBCs is the dysfunction of Na+, K+ ATPase which is reversible with the blood glucose levels.[67] These cells are also less deformable, slow to recover their original shape and have a higher tendency to aggregate when compared to non-diabetic patients.[68–72]. The

loss of deformability correlated with the extent of glycemic control, that is, formation of glycosylated hemoglobin.[73–75] At the cytoskeletal level, β spectrin, actin and protein 4.2 are found to be glycosylated leading to their oxidative damage[76,77] contributing to the cell stiffness. Increase in nitric oxide and viscosity of blood plasma, as observed in DM patients, when combined with the less deformable RBCs leads to vascular complications such as atherosclerosis.[78,79]

4.3 RBCs and Senescence

RBCs do not undergo autophagy as they lack the required cellular machinery. They are instead removed from circulation by macrophages in a process termed erythrophagocytosis. As they age, RBCs accumulate certain markers on them which enables their selection and uptake by macrophages.[80,81]

One of the markers of RBC senescence is loss in deformability.[82] This results in them being filtered out of circulation by the inter-endothelial slits and phagocytosed in the red pulp of the spleen. The cytoskeletal changes resulting in this loss of deformability can be attributed to multiple pathways. As they age, senescent RBCs (sRBCs) accumulate increasing amounts of irreversibly oxidized proteins/hemoglobin[83] leading to an increase in their density.[84,85] There is an increase in membrane bound hemoglobin which triggers an increase in ROS within the cell.[86,87] Within the membrane, hemoglobin binds to the protein, band 3, leading it to cluster.[88–90] Band 3 is an integral membrane protein which also functions as a surface antigen for immunoglobulin antibodies. These antibodies mark cells to be phagocytosed. On the cytoplasmic side band 3 protein is a site for cytoskeleton and membrane linkage via the protein ankyrin.[91,92] Hence, a band 3 cluster formation disrupts the cytoskeleton and lipid membrane linkages. In addition, there are reports of in vivo crosslinking of hemoglobin to spectrin which also contributes to increased cell rigidity with aging.[86,93]

5 Summary

It can be seen that deformability affects all aspects of RBC health and functioning. The morphology of the RBC is such as to impart the cell high deformability, the cell metabolism is directed towards maintaining it and the disease states and senescence progress by affecting it. Many studies have therefore focused on understanding the pathways affecting RBC deformability. A knowledge of this can help in developing potential techniques aimed at restoring it.

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Compliance with Ethical Standards

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Ms. Shivangi Paradkar is a Project Associate at the Centre for BioSystems Science and Engineering at Indian Institute of Science (IISc), Bangalore. Prior to IISc, she was associated with IIT Hyderabad as a Junior Research Fellow, working on 'Nanoparticle based Cancer Theranostics'. Additionally, she has also worked at Centre for Cellular and Molecular Biology (CCMB), Hyderabad, on 'Paper Microfluidics', which happens to be her masters dissertation. She has been involved, both professionally and academically, in diverse fields of biotechnology, exploring the various aspects of biological systems.

Dr. Priya Gambhire is a DST-Inspire Faculty Fellow at the Center of Biosystems Science and Engineering at IISc. Prior to joining the department in 2017, she did her postdoctoral research at Prof. V. Kumaran's laboratory in IISc and Dr. Annie Viallat's group in CINAM (CNRS), Marseille France. Her PhD is from Prof. Rochish Thaokar's group in IIT Bombay in 2012. Her research theme is to study biological flows using engineering principles most often using microfluidic tools. She currently pursues theoretical and experimental studies on red blood cell deformability and endothelial cell mechanics during flow.