

Chapter 2

Nano-Mechanical Response of Red Blood Cells

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Abstract In their physiological function, red blood cells (RBCs) need to undergo large deformations in order to pass through capillaries and small vessels. In several pathological conditions, including diabetes mellitus and Alzheimer's disease, this extreme deformability appears to be deeply impaired and an increase in the RBCs stiffness is usually detected.

Given the key role played by the mechanical properties of RBCs, we investigated their viscous-elastic response by AFM nano-mapping. High-resolution maps demonstrate that healthy erythrocytes are stiffer in their center and softer at the periphery. The RBC stiffness profile shows a cylindrical symmetry that appears to be strongly correlated with their typical biconcave shape.

Our measurements show that the Young's modulus is strongly depending on the indentation rate, demonstrating that viscous forces have a key role in determining their mechanical response. The importance of viscous forces is further stressed by the comparison between healthy and pathological erythrocyte. Our data show that pathological RBCs are not simply stiffer than healthy ones. Conversely they display a different dependence on the indentation rate that leads to an apparent increase in stiffness. Taken together our results show that both the local stiffness distribution and the viscoelastic response provide important information on RBC biomechanics.

Keywords Atomic force microscopy • Stiffness • Dissipation • Biomechanics • Red blood cells

2.1 Introduction

Red blood cells (RBC) have a typical biconcave shape with dimensions of approximately 8 μm in diameter and 2 μm in thickness and contain an interior viscous liquid enclosed by a viscoelastic membrane [1–7]. Such membrane consists of a nearly incompressible lipid bilayer attached to a spectrin protein network, held together by short actin filaments [7].

The peculiar RBC membrane structure ensures the integrity of the cell in narrow capillaries whose cross-section is smaller than the size of the cells. Under physiological conditions, indeed, RBCs must undergo repeated and severe deformations when travelling through small capillaries with diameter not more than 3–5 μm . Moreover, when passing through the spleen RBCs are required to traverse extremely narrow slits with sizes less than 1 μm [8].

There is a growing evidence that this extreme deformability is significantly altered in pathological conditions, such as diabetes mellitus, essential hypertension, arteriosclerosis and coronary artery disease [5, 9, 10]. On the other hand, an altered red blood cell deformability may contribute to the onset and the development of many pathologies. For example, membranopathies and hemoglobinopathies are known to alter the RBC deformability, affecting the blood flow in large and small vessels [11–13].

On the one hand, RBC modifications occur at the whole cell level, on the other hand they are closely related to changes in the molecular composition and organization of the cell that, in their turn, occur at the nanoscale.

This has made it necessary to develop quantitative tools able to probe RBC changes at nanometer and piconewton scales. In this regard, Atomic Force Microscopy (AFM) is an extremely powerful technique as it permits to probe cells, tissues and molecule at the nanoscale in nearly physiological conditions [6, 14–25].

One of the key characteristics to look at RBC biomechanical properties by AFM is the Young's modulus (E) that provides information on cell stiffness. The nanoscale mapping of E has been proven to be effective in distinguishing between normal

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and pathological erythrocytes in several disease conditions [6–9, 17–19], opening the way to the development of novel diagnostic tools.

Many biomechanics AFM studies rely on the basic assumption that RBCs behaves like an elastic body: dissipative forces are neglected and Young's modulus is treated as unaffected by probe dynamics. As recently demonstrated in [6], this assumption cannot be considered strictly valid and dissipative forces play a key role in the biomechanical response of red blood cells at the nanoscale level. This result is in close agreement with the peculiar structure of the RBC membrane that can be modelled as a network of viscoelastic springs mediating elastic and viscous response.

One of the major properties to evaluate quantitatively the contribution of dissipative forces is to measure the percentage of energy dissipated during the AFM indentation process or Hysteresis (H). Hysteresis is rarely considered in AFM experiments on the biomechanical behaviour of red blood cells. Nevertheless, the nanoscale mapping of H can provide valuable information on the RCB modifications occurring at the molecular level at the onset of many pathologies.

In this work we describe a novel scanning probe-based nanoscale mapping methodology that generates Hysteresis maps of cells and tissues, which have been subjected to AFM indentation.

To test this method we used red blood cells extracted from an healthy donors and patients with iron overload and hyperferritinemia, showing that H may provide highly valuable information with potential application in the clinical practice.

2.2 Material and Methods

The blood was obtained from healthy donor volunteers and patients with iron overload and hyperferritinemia, anticoagulated with heparin and centrifuged to separate blood form serum. Erythrocytes were than dissolved in physiological solution and deposited on a poly-L-lysine coated petri dish. After 1 h incubation, the poly-L-lysine coated petri dish was gently washed in physiological solution to remove unattached red blood cells.

Subjects affected by iron overload and hyperferritinemia were selected because increased serum ferritin concentration is associated with inflammation processes, which lead to a number of changes in the biophysical properties of red blood cells, including aggregation, sedimentation and deformability [2, 26].

Measurements were performed at 37 °C in liquid environment using a JPK Nanowizard II atomic force microscope (JPK instruments, berlin Germany) coupled to an optical microscope (Axio observer, Carl Zeiss, Milan, Italy). MikroMasch silicon cantilevers with a spring constant of approximately 0.05 N/m and a tip radius of about 10 nm were used (CSC38, MikroMasch). The cantilever spring constant was computed for each measurement by thermal calibration. Force curves were acquired by using an indentation force of 0.5 nN.

To evaluate quantitatively the contribution of dissipative forces, we estimated the energy dissipated during the deformation process (or Hysteresis H). H was computed as the difference between the area (A_E) under the extension force curve $F_E(\delta)$ and that (A_R) under the retract curve ($F_R(\delta)$) normalized by A_E :

$$H = \frac{\int_0^\delta F_E(\delta) d\delta - \int_0^\delta F_R(\delta) d\delta}{\int_0^\delta F_E(\delta) d\delta} = \frac{A_E - A_R}{A_E} \quad (2.1)$$

The nanoscale mapping of H was obtained by a homemade Labview software that allows us for the contemporaneous determination of hysteresis and work of adhesion.

2.3 Results

In Fig. 2.1a, b two representative approach-retract cycles acquired on a healthy (Fig. 2.1a) and a pathological RBC are shown (Fig. 2.1b). The coloured area between the approach and the retract curve provides a graphical representation of H.

One can observe that the pathological red blood cell displays a larger H value than the healthy one. However, the typical biconcave shape of red blood cell hints at a spatial inhomogeneous biomechanical response, suggesting that a single point measure could not be representative of the overall behaviour of the cells.

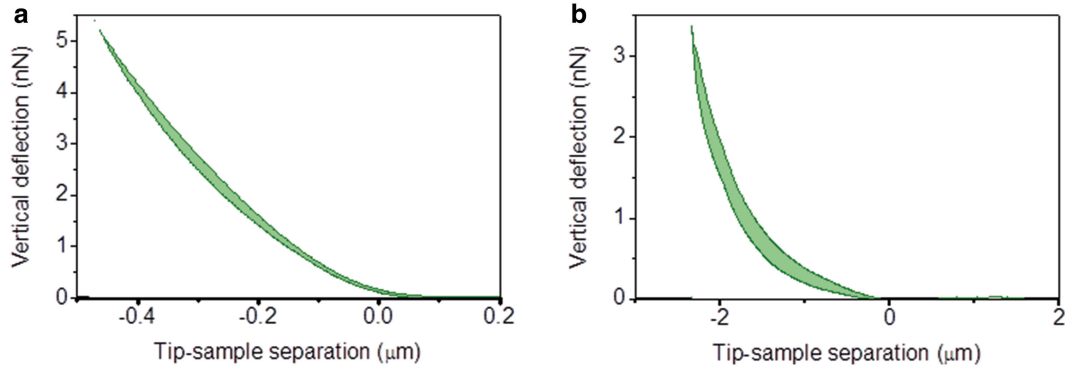


Fig. 2.1 Two representative approach-retract cycles acquired on healthy (a) and pathological (b) RBCs. The green coloured area of the cycle represent the hysteresis

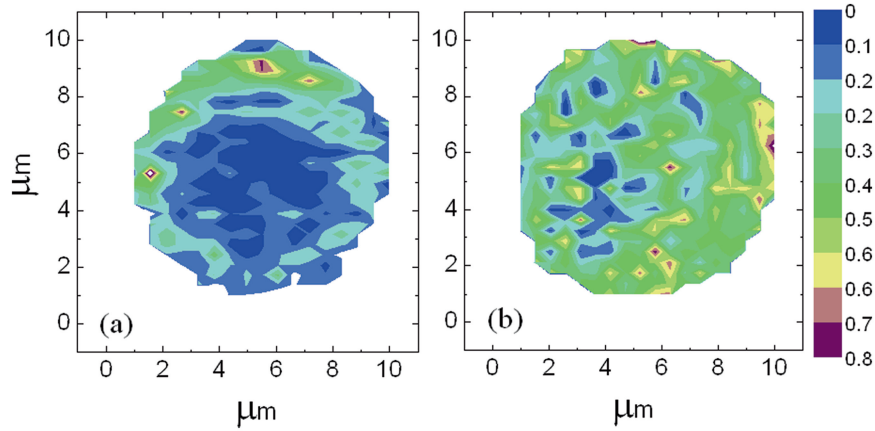


Fig. 2.2 High-resolution hysteresis maps of healthy (a) and pathological (b) RBCs. Both maps are represented with the same colour scale. These maps display the presence of a cylindrical distribution of H for the healthy RBC, correlated with the biconcave shape of the cell, and the lack of this spatial symmetry for the pathological one. Indeed normal RBC is characterized by value of H in the range 0–0.1 in the centre, and an increase of H in the periphery, while the pathological erythrocyte shows higher value of H, uniformly distributed

As far as RBC stiffness is concerned, this hypothesis was recently confirmed in Ref. [6]. The high resolution nanoscale mapping of E values acquired in physiological solution unveiled that healthy erythrocytes are stiffer in their centre and softer at the cell periphery [6].

Therefore we probed the local response of RBCs by acquiring force-distance curves at different positions over the cell surfaces and evaluating the perceptual of energy dissipated during the indentation process, through the estimation of H (Eq. (2.1)).

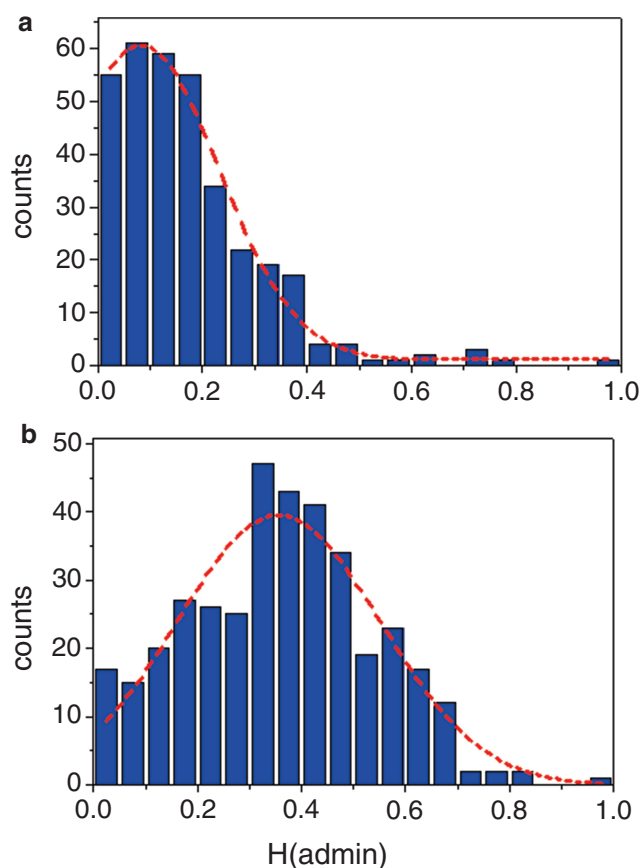
In Fig. 2.2a, b we reported two hysteresis maps acquired on an healthy (a) and pathological red blood cell (b). H values range between 0 and 0.8 for both cells, indicating that a perceptual energy ranging from 0 to 80 % is dissipated during the indentation process.

As far as the healthy RBC is concerned, the nanoscale mapping of H shows a cylindrical symmetry. The cell centre behaves approximately as a pure elastic body, showing H values ranging from 0 to 0.1. An increase in H values can be observed at the cell periphery, where H can be as high as 0.8.

The cylindrical distribution of H values appears to be strongly correlated with the typical biconcave shape of healthy red blood cells. Moreover such distribution well correlates with the Young's modulus distribution detected in healthy red blood cells [6].

A significantly different result is obtained in the pathological case. In this case, the nanoscale map appears to be homogeneously brighter than that of the healthy RBCs indicating that a larger amount of energy is dissipated during the indentation process. Moreover, the cylindrical distribution of H values on healthy RBCs is not observed. Conversely, an almost uniform distribution can be detected.

Fig. 2.3 Histograms of H value for the healthy (a) and pathological (b) RBCs. Data are fitted with a Gaussian curve (red dashed line). The distribution of the healthy cell is peaked at 0.08, while distribution's peak of the pathological cell is shifted to 0.35



A frequency histogram of H values is reported in Fig. 2.3a, b for the healthy and pathological RBC, respectively. A Gaussian curve was fitted to data (red dashed line).

It can be clearly noted that frequency distribution for the healthy RBC has a peak at approximately 0.08. An average value of 0.17 ± 0.14 can be measured (data are expressed in terms of mean $\pm$ standard deviation of the mean). A clear shift of the frequency distribution at larger H values can be observed in the pathological case where a peak at approximately 0.35 can be detected. An average value of 0.35 ± 0.19 can be estimated (data are expressed in terms of mean $\pm$ standard deviation of the mean).

2.4 Discussion

The Atomic Force Microscopy is a widely used surface microscopy technique capable to reconstruct the topography of materials and biological samples at the nanometer resolution under virtually any environmental conditions [21, 27–31]. This unique characteristic makes the AFM a key tool to perform high resolution images of cells and tissues in their natural state. Moreover AFM can be used to probe the biomechanical response of biological specimens at both the microscale and the nanoscale level [5, 6, 14–24, 31].

One of the major properties to look quantitatively at the mechanics of biological systems is Young's modulus E, as measured by the analysis of AFM force-distance curves. Mapping E values at the nanoscale level, indeed, has proven to be a valuable parameter with potential application in the clinical practice, in particular in diagnostics [4–6, 31, 32].

Beside Young's modulus, AFM can provide other biomechanical parameters such as adhesion, relaxation time and hysteresis. The latter is rarely considered in AFM experiments on biological samples. Nevertheless it can provide valuable insights into the molecular modifications occurring during the genesis and the development of many pathologies [6, 33].

In this work we developed a novel scanning probe based methodology that, through mapping, permits to investigate the role of dissipative forces on the whole cell mechanics. The method was tested by using RBCs extracted from healthy donors and patients with hyperferritinemia and iron overload.

Our high resolution hysteresis maps acquired in physiological solution unveil the local changes in the biomechanical response of red blood cells, demonstrating that healthy erythrocytes behaves approximately like an elastic body as far as the cell centre is concerned. Conversely, the cell periphery appears to be more dominated by dissipative forces. This result might be particularly relevant for the theoretical modeling of red blood cells motion under a flowing condition, as it suggests to model the cell centre by using mainly network of elastic springs and the cell periphery by using viscoelastic responders.

The measured spatial distribution of H values on the surface of healthy RBCs shows a cylindrical symmetry that appears to be strongly correlated with the typical RBC biconcave shape. These results hint at the presence of a close relation between the biconcave shape of healthy red blood cells and their mechanical properties. Moreover the spatial inhomogeneity of H values further stresses that a single point measure (as well as some random-landing measures) often used in AFM experiment cannot be representative of the whole RBCs mechanical response.

Healthy red blood cells display an extremely low average H value (0.17 ± 0.14), hinting at a nearly elastic behavior. This finding deserves a more in-depth study as it might have a functional role. In physiological conditions indeed, RBCs undergo extreme deformations when passing through small vessels and capillaries. Subsequently they easily recover their original shape, suggesting the absence of plastic deformation that might reflect in an increased H value.

Such behaviour appears to be significantly modified in the case of pathological red blood cells, where larger H values were measured (0.35 ± 0.19). This impaired deformability is highly interesting towards a better comprehension of the molecular mechanism underlying the RBC shape, roughness and mechanical modifications occurring at the onset of many pathologies. Our nanoscale mapping highlighted that not only the average H value is changed, but also its spatial distribution. The cylindrical distribution of H values is indeed not observed in the pathological case, hinting at the occurrence of deep structural modifications in the RBC ultrastructure.

The observed changes in H values and their spatial distribution might be due either to changes in viscoelastic properties of the cell membrane or to a modified viscosity of the inner cytosol. Iron overload conditions can indeed be related to an increased haemoglobin (Hb) concentration that might lead to an augmented inner medium viscosity. At the same time the presence of unbound iron causes lipid oxidative stress and consequently impairs the cell membrane. Both phenomena can be linked to the observed modification of the hysteresis map. However, as the major changes occur within the centre of the cell, we suppose that H variation might be mainly related to membrane properties, rather than to a homogeneous change in the inner medium viscosity. A possible method to distinguish between membrane and inner medium contribution would involve the study of the dissipative response of RBC ghosts (RBC membrane lipid bilayer with cytoskeleton), that would help us to avoid the contribution due to the inner medium.

Taken together, our results highlight the importance of mapping the local distribution of hysteresis in the biomechanical characterization of cells. The nanoscale mapping of H indeed permits to clearly distinguish between healthy and pathological red blood cells paving the way to the development of novel diagnostic tools based on the measurement of hysteresis as probed by AFM indentation cycles.

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