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Structural and Mechanical Analysis of Erythrocyte Membranes Using Atomic Force Microscopy

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ABSTRACT

Red blood cells (RBCs) are crucial for the transportation of oxygen throughout the body and the removal of carbon dioxide. Understanding the structure and mechanical properties of RBC membranes is critical for identifying potential diseases associated with membrane dysfunction. Atomic force microscopy (AFM) is an effective technique for investigating the nanoscale features of erythrocyte membranes, providing high-resolution imaging and precise mechanical property data. This article focuses on the experimental approach used to investigate the membrane structure of erythrocytes and the insights gained. AFM was used to assess the morphology and elastic properties of blood cells, with quantitative measurements of the cell membrane's elastic modulus performed using force spectroscopy. The elastic modulus was observed to change based on both the location of the indentation and the duration of contact with the membrane. Furthermore, a significant relationship was identified between the indentation rate and the estimated elastic modulus.

Keywords: Modulus of elasticity, Erythrocyte, Force spectroscopy, Atomic force microscopy

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Introduction

Atomic force microscopy (AFM) enables surface imaging with a resolution approaching atomic dimensions [1] and is widely used to evaluate the local elastic and adhesive properties of surfaces [2]. Despite its effectiveness, the use of AFM to study biological samples presents challenges in both experimental techniques and data interpretation. Overcoming these challenges yields valuable insights into membrane topography and mechanical properties, as well as changes in the cytoskeleton, motor protein dynamics, and other essential cellular activities [3, 4]. To date, AFM has been applied to imaging DNA, RNA, bacteria, viruses, tissues, and even organs [5-7].
This study focuses on erythrocytes, which are approximately 7.2–7.5 μm in diameter, typically exhibiting a deviation of less than 0.5 μm, and 2 μm in thickness. The concentration and properties of erythrocytes, such as deformability and aggregation tendencies, are essential factors determining blood rheology [8, 9]. Erythrocyte deformability is crucial for their ability to transport substances through the circulatory system, including capillaries as narrow as 2 μm in diameter [10]. According to Alexy *et al.* [10], disruptions in hemorheology lead to impaired blood flow and decreased transport efficiency within the microcirculatory system. Hence, evaluating

erythrocyte rheological properties plays a vital role in diagnosing diseases and enhancing therapeutic approaches [11, 12].

Existing clinical techniques assess erythrocyte deformability and aggregation in suspension but fail to evaluate individual cell characteristics [13, 14]. Atomic force microscopy offers a more precise method, allowing detailed analysis of individual erythrocyte shape, membrane structure, and elasticity. Through force spectroscopy, AFM can quantify the elastic modulus at the local level with nanometer precision [15, 16].

The method for evaluating the elastic properties of materials involves measuring resistance to deformation by applying a rigid indenter. For elastomers (viscoelastic polymer materials), deformation behavior is largely influenced by the rate of deformation and the time of contact [17]. These factors complicate the determination of mechanical properties of biological membranes, as values are dependent on the indenter's speed and the duration of contact [18].

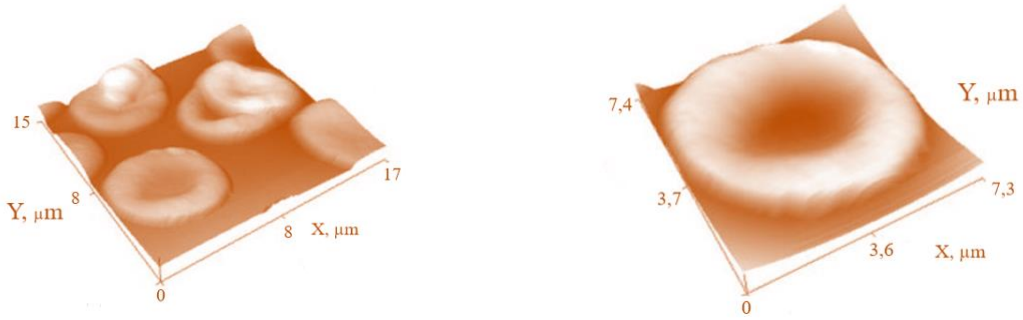
Erythrocytes were chosen for this study because they are simple and readily accessible, with membranes that exhibit common characteristics shared by biological membranes [19]. Thus, erythrocytes serve as an ideal model for developing cell elastography techniques and studying the mechanical properties of membranes and their changes in various pathological conditions [20].

This work aims to investigate the application of atomic force microscopy in assessing the elastic and viscoelastic properties of erythrocyte membranes.

Materials and Methods

The study utilized blood samples from individuals without significant acute or chronic conditions. A venous blood sample, which was stabilized with heparin, was mixed with a 1.5% glutaraldehyde solution for fixation. The erythrocytes were then isolated from the plasma by centrifugation at 1500 rpm for three minutes. Afterward, the erythrocytes were washed three times with a buffer solution and three more times with distilled water. The final erythrocyte sample was applied onto 10 × 10 mm glass slides and left to air-dry at room temperature.

For examining the geometry, membrane surface, and elasticity of the erythrocytes, a specialized setup combining atomic force microscopy (AFM) and optical microscopy was employed. This system incorporated an NT-206 AFM, capable of micro-positioning the probe across a 10 × 10 mm area, along with an optical imaging system. AFM scanning was conducted using standard silicon probes (NSC11, MikroMasch Co., Estonia) in contact mode, capturing both the topographic images and lateral force measurements. The first step of the procedure involved recording an optical image of the cells on a glass surface. A mechanical micropositioning system was used to locate the measurement area, with the probe's position being verified through optical control. The scanning area was reduced from 30 µm to 0.5 µm using a piezoelectric AFM scanner, as shown in **Figure 1**.



a b

Figure 1. Spatial AFM image of erythrocytes: a) is the scanning area of 15 × 17 µm; b) scan area of 7.4 × 7.3 µm

The evaluation of the cell membrane's elastic properties was carried out using the static force spectroscopy technique [21]. This function is a standard mode in atomic force microscopy. The principle behind this method involves applying contact deformation to the studied object using the probe tip and measuring the interaction force between the probe and the surface as a function of the distance between them [22]. During the static force spectroscopy process, the probe does not oscillate but remains stationary at the anchor point.

For the static force spectroscopy measurements, a silicon probe was used after its tip was slightly "blunted." The radius of the tip's rounded end was approximately 60 nm, determined by scanning a reference sample, and the probe's stiffness coefficient was 3 N/m, as specified by the probe manufacturer.

Results and Discussion

By scanning the sample, we obtained images showcasing the surface topography of the erythrocyte membranes, along with the three-dimensional structure of the red blood cell cytoskeleton, which can be distinguished from the lateral force images. **Figure 2** illustrates a segment of the membrane surface with dimensions of 1.8 × 1.8 μm². Scanning areas of the membrane within the 1-2 μm range allows for the assessment of both its structure and the identification of the cytoskeleton's near-surface arrangement. The cytoskeletal mesh pattern is more clearly visible when using the lateral force mode [23]. The typical size of the cytoskeleton elements is approximately 50-70 nm.

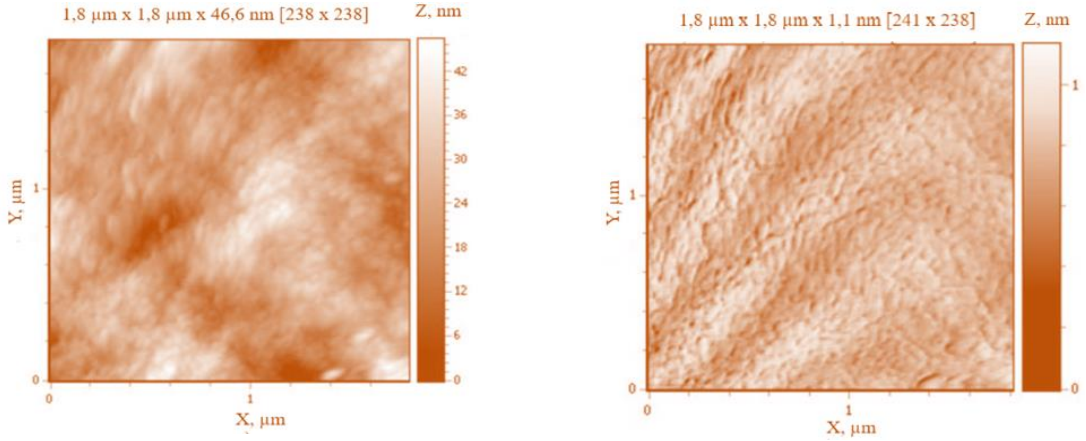


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Figure 2. Structure of the erythrocyte membrane surface: a) topography mode, and b) lateral forces mode

AFM provides an effective tool to assess the morphology and structural features of cell membranes, particularly in pathological conditions. In addition to this, the technique allows for the evaluation of the local mechanical properties of cells. One of the primary considerations when analyzing the elastic modulus is the cell's thickness at the point of indentation. For erythrocytes, the thickness of the membrane at the cell's edge is much greater than in the center; choosing the probe's insertion area is critical to ensure accurate results. The interaction between the cell and the rigid substrate can affect the elasticity measurements, but this effect is minimal if the indentation depth does not exceed 10% of the cell's thickness. In our experiment, the indentation depth was kept < 7 nm, representing only 0.4% of the peripheral and 1.4% of the central thickness of the erythrocyte membrane.

The force spectroscopy measurements were taken one hour after preparing the samples. Five healthy individuals' erythrocytes were analyzed, with ten cells per donor, and three probes were inserted into both the peripheral and central parts of each cell. The elasticity modulus was measured at varying depths of probe penetration, from 1 nm to 7 nm. For each sample, the modulus values were averaged, and the deviation was recorded. Statistical graphs were created to show the relationship between the local modulus and indentation depth. A nonlinear correlation between indentation depth and the elastic modulus was observed. Interestingly, significant differences between the peripheral and central parts of the membrane were only noted at a depth of 3.12 ± 0.029 nm. Overall, within two hours of preparation and using probes with a curvature radius greater than sixty nm, the results were not influenced by the insertion site of the probe.

To evaluate the influence of loading time on the cells' elasticity, measurements were taken at different speeds (300, 30, 15, and 1 nm/s) of the sample stage. Indentation tests were performed in air, one hour post-preparation, at the periphery of the erythrocyte. The amount of bending of the probe was recorded at various speeds. The results showed that as the loading speed decreased, the erythrocyte membrane's resistance to deformation increased. This was reflected by a rise in the calculated elastic modulus and a decrease in the maximum depth of probe insertion achievable with the selected probe stiffness. Specifically, at a probe penetration depth of 5 nm, the Young's modulus at a speed of 1 nm/s was found to be 4.5 and 15.6 times greater than at thirty nm/s and 300 nm/s, respectively. These results indicate a clear dependency of the erythrocyte's elastic properties on the rate of deformation, emphasizing the viscoelastic behavior of the membrane. These findings are consistent with earlier studies using optical tweezers to examine the mechanical properties of individual erythrocytes, which also demonstrated viscoelastic characteristics under substantial elastic deformation.

The assessment of the structural and mechanical characteristics of red blood cells suggests that, provided the substrate's influence is removed, the choice of indentation location is not critical for these cells. This condition holds as long as the probe's insertion depth remains less than 10% of the cell's thickness. Additionally, the modulus of elasticity has been shown to vary with probe penetration depth, which may be attributed to the heterogeneity of the studied objects and the influence of changes in lateral tension of the membrane, which occurs in response to the deformation of the lipid bilayer.

Furthermore, a clear correlation between Young's modulus and loading time was found in erythrocytes. However, the underlying mechanism driving this behavior of the red blood cell membrane remains unclear.

It is important to note that the mechanical properties of the cells were examined in air, after they were dried onto a substrate. This drying process inevitably resulted in reduced membrane elasticity, increased resistance to deformation, and, consequently, a higher measured elastic modulus. Moreover, it is essential to employ a more appropriate model for contact deformation that better describes the experimental setup for loading cells and accounts for their shell-like structure. Therefore, the elastic modulus values obtained should be viewed as estimations, with the resulting relationships being more qualitative than strictly quantitative.

Conclusion

The studies carried out highlight the specific methodological aspects of using atomic force microscopy, with a focus on the application of static force spectroscopy to assess the local mechanical properties of cells. The findings emphasize the importance of selecting the proper rate of contact between the indenter and the erythrocyte membrane when evaluating its elasticity. Based on the results, it is evident that the mechanical properties of the membrane are significantly influenced by the interplay between relaxation transitions and the temporary exposure time during the testing process. The viscoelastic nature of the cells remains a subject for further investigation, as the observed direct correlation between the elastic modulus and loading rate is not yet fully understood.

The results obtained for the mechanical properties of the erythrocyte membrane can vary greatly due to inconsistent measurement conditions, which complicates the process of quantifying and comparing findings. For reliable and comparable outcomes, strict adherence to experimental protocols and transparency in the methodology used to calculate the mechanical properties is essential.

With proper regulation of experimental conditions, atomic force microscopy offers the potential for accurate and quantitative assessment of cellular mechanical properties, opening avenues for research at the nanometer scale and advancing the field of cellular elastography.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: The study used blood from individuals without any major acute or chronic diseases. All participants provided informed consent for voluntary involvement in the research.

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