Original Article

Study of the Structural and Mechanical Properties of Erythrocyte Membranes Using Atomic Force Microscopy

Akhmad Ramanovich Orusbiev[1], Tamila Gajibutayevna Alunkacheva[2], Maryana Sabirullaevna Charandaeva[2], Bella Saidaminovna Kireeva[3], Magomedali Feizulaevich Gadzhiev[2], Vladislav Gennadevich Zelenetckii[4*]

[1]Department of Therapy, Medical Faculty of Saratov State Medical University named after Razumovsky, Saratov, Russia. [2]Department of Therapy, Medical Faculty of Dagestan State Medical University, Makhachkala, Republic of Dagestan, Russia. [3]Department of Therapy, Faculty of Medicine of the Chechen State University, Grozny, Republic of Chechnya, Russia. [4]Department of Therapy, Medical Faculty of the Medical Institute of Tula State University, Tula, Russia.

Abstract

Red blood cells (erythrocytes) are one of the most common cells in the human body and are responsible for transporting oxygen to tissues and removing carbon dioxide from the body. The study of the structure of erythrocyte membranes is of great importance for understanding their functions and the possibility of detecting various pathological conditions associated with a violation of the mechanical properties of membranes. Atomic force microscopy is a powerful tool for studying the nanostructural properties of membranes and allows the obtaining of high-quality images and data on mechanical properties. This article describes an experimental technique used to study the structure of erythrocyte membranes, as well as the results obtained and their analysis. The morphology and elastic properties of blood cells were analyzed by atomic force microscopy. Quantitative estimates of the elastic modulus of the cell membrane in the mode of force spectroscopy have been performed. The values of the elastic modulus of erythrocytes were determined depending on the localization of the indentation area and the time of exposure to the membrane surface by the probe. A significant dependence of the results of the elastic modulus estimation on the rate of indenter action on the cell membrane is shown.

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

Introduction

Atomic force microscopy (AFM) is one of the tools that provide a spatial image of the surface with a resolution close to atomic [1]. In addition, AFM is successfully used to quantify the local elastic and adhesive properties of the surface [2]. When studying biological objects using AFM, certain difficulties arise in implementing techniques and interpreting data, overcoming which allows us to obtain unique information about the topography of the membrane surface and its mechanical properties, as well as about changes in the structure of the cytoskeleton during cell movement, about the movements of motor proteins and other fundamental cellular processes [3, 4]. Currently, AFM images of DNA, RNA, bacteria, and viruses, as well as tissues and even organs have been obtained [5-7].

As the object of this study, erythrocytes were selected – cells having the shape of a biconcave disc with a diameter of 7.2–7.5 μm with deviations in both directions for most no more than 0.5 μm and a thickness of 2 μm. The concentration of these shaped blood elements and their properties (deformability, tendency to aggregation) are among the factors that largely determine the rheological properties of blood [8, 9]. Thus, the ability of erythrocytes to deform is the most important rheological phenomenon that allows these cells to deliver substances necessary for the vital activity of the body through the vascular system, including capillaries whose diameter reaches 2 μm [10]. The results of Alexy *et al*. (2022) indicated that changes in hemorheology lead to a violation of blood flow and a decrease in the efficiency of the transport function of the microcirculatory system [10, 11]. Therefore, the assessment of the rheological properties of blood cells is important from the point of view of both identifying the features of the course of diseases and improving the effectiveness of treatment [12].

Currently, in clinical practice, integral methods are used to determine the deformability and ability of erythrocytes to aggregate in suspension, which does not allow the assessment

Address for correspondence: Vladislav Gennadevich Zelenetckii, Department of Therapy, Medical Faculty of the Medical Institute of Tula State University, Tula, Russia.
ruslankalmykov777@yandex.ru

How to cite this article: Orusbiev AR, Alunkacheva TG, Charandaeva MS, Kireeva BS, Gadzhiev MF, Zelenetckii VG. Study of the Structural and Mechanical Properties of Erythrocyte Membranes Using Atomic Force Microscopy. Arch Pharm Pract. 2023;14(2):70-4. https://doi.org/10.51847/yGaXHi9JBR

of the state of an individual cell [13, 14]. Such estimates are possible using atomic force microscopy, which allows us to study the shape of an individual cell, the topography of its membrane surface, and its elasticity. In particular, using the power spectroscopy function, it is possible to locally estimate the modulus of elasticity when an indenter is exposed to a cell area up to several nanometers [15, 16].

The method of assessing the elastic properties of materials in surface layers consists in measuring their ability to resist the introduction of a rigid indenter with a given load. It is known that the deformation behavior of elastomers (viscoelastic polymer materials) is largely determined by the time and rate of deformation [17]. This determines methodological difficulties in determining the mechanical characteristics of biological membranes: it is difficult to create reference samples since measured values of the characteristics of biological objects depend on the speed of movement of the indenter during loading and the time the indenter is in contact [18].

The choice of erythrocytes as the object of this study is due to the cells being relatively simple and easily accessible, and at the same time, erythrocyte membranes are characterized by general principles of the organization of biological membranes [19]. Therefore, it is convenient to use them in the development of cell elastography techniques as a natural model for studying the general structural and functional characteristics of membranes, as well as changes in their mechanical properties in various diseases [20].

The purpose of this work is to determine the features of the use of atomic force microscopy to assess the elastic and viscoelastic properties of the biological membranes of erythrocytes.

Materials and Methods

The blood of individuals without any serious acute and chronic diseases was used for the research. A drop of venous blood previously stabilized with heparin was fixed in a test tube with a 1.5% solution of glutaraldehyde. The erythrocytes were then separated from the plasma by centrifugation for 3 min at 1500 rpm. The resulting erythrocyte sediment was washed three times from the fixative in a buffer solution, and then three times in distilled water. After that, the erythrocytes were applied to 10×10 mm slides and dried in air at room temperature.

The study of the geometry, surface structure, and elastic properties of the erythrocyte membrane was carried out using a specialized experimental complex combining the functions of scanning probe and optical microscopy.

This complex consists of an NT-206 atomic force microscope with the capabilities of micropositioning the probe above the sample within a 10×10 mm area and an optical system. AFM scanning was performed with standard silicon probes NSC11 (MikroMasch Co., Estonia) in contact mode with simultaneous registration of images of topography and lateral forces. In the experimental study, an optical image of cells on a glass substrate was initially recorded. Then, with the help of a mechanical micropositioning system, an area for AFM measurements was selected during optical control of the probe position in the sample plane. Further reduction of the visualization area of the object within the characteristic scan size from 30 to 0.5 µm was carried out using a piezoelectric AFM scanner **(Figure 1)**.

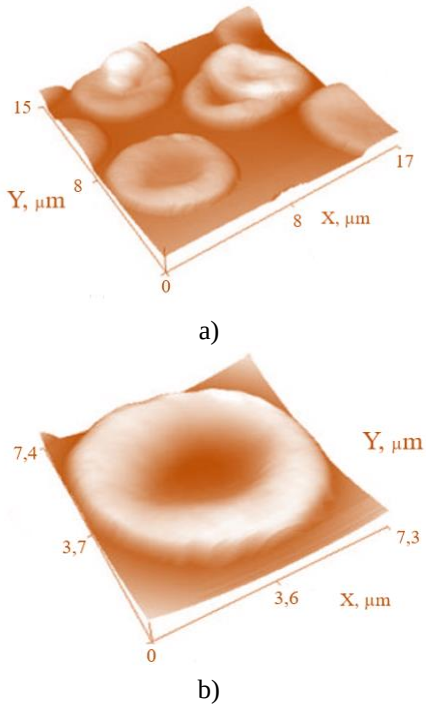

Figure 1. Spatial AFM image of erythrocytes: a) is the scanning area of 15×17 µm; b) scan area of 7.4×7.3 µm

The method of static force spectroscopy was used to evaluate the elastic properties of the cell membrane [21]. The power spectroscopy function is the standard operating mode of an atomic force microscope. The essence of the method consists of the implementation of contact deformation of the object under study by the tip of the probe and in measuring the dependence of the interaction force of the probe with the sample surface on the distance between them [22]. When implementing the static force spectroscopy procedure, the probe console does not perform forced oscillations and occupies a static position at the anchorage point.

The static force spectroscopy procedure was performed with a silicon probe after some "blunting" of the tip. The radius of rounding of the tip was about 60 nm (determined by scanning a test sample), and the stiffness coefficient of the console was 3 N/m (according to the specification of the probe manufacturer).

Results and Discussion

As a result of scanning the sample, images of the topography of the surface of erythrocyte membranes, as well as the spatial structure of the cytoskeleton of red blood cells were obtained in contrast to the image of lateral forces. **Figure 2** shows a section of the membrane surface measuring 1.8×1.8 μm^2. It is when scanning sections of the cell membrane with a size of 1-2 μm that it is possible to assess its structure, as well as to detect the near-surface structure of the cell cytoskeleton. The mesh structure of the cytoskeleton is more clearly manifested in the mode of lateral forces [23]. The characteristic size of the cells formed by the elements of the cytoskeleton is 50-70 nm.

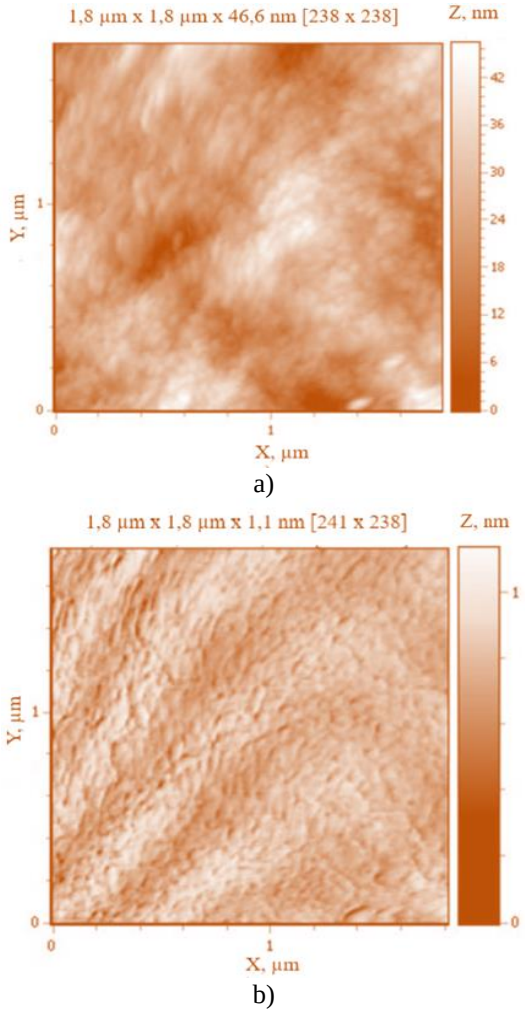

Figure 2. Structure of the erythrocyte membrane surface: a) topography mode; b) lateral forces mode

Thus, AFM images allow us to compare the morphology of cells, as well as to evaluate the structural features of their membranes, in some pathologies.

Another important possibility of AFM analysis of a cell is the assessment of its local mechanical properties. The use of force spectroscopy techniques in this case has several fundamental features. One of the factors that must be taken into account when analyzing the absolute values of the elastic modulus is the thickness of the cell at the indentation point. Therefore, when studying the elastic properties of erythrocytes, it is important to determine whether the choice of the probe insertion area affects the determined mechanical properties of the cells since the thickness of the cell at the periphery significantly exceeds the thickness of the central part. The effect of a rigid substrate on the value of the estimated modulus of elasticity depends on the ratio between the depth of indentation and the thickness of the cell. It is believed that this effect can be neglected if the depth of indentation does not exceed 10% of the thickness of the object under study. In our experiment, the penetration depth of the probe was comparable to the thickness of the cell membrane and did not exceed 7 nm, which was 0.4% of the peripheral and 1.4% of the central thickness of the erythrocyte.

The power spectroscopy procedure was performed in air, one hour after the sample preparation. The erythrocytes of five healthy individuals were examined. Measurements were carried out on ten different cells for each donor as three probes were inserted into the central part and the same number into the peripheral part of the cell. The value of the elastic modulus was estimated at different depths of indenter insertion, sequentially from 1 nm to 7 nm. In the statistical evaluation of the local modulus of elasticity, reference points were selected, for which the average value of the modulus of elasticity and deviation from the average was found. Statistically averaged graphs of the dependence of the local modulus of elasticity on the depth of indentation for various regions were constructed for the selected points. It is established that the dependence of the elastic modulus on the depth of indenter insertion is nonlinear. A comparative analysis of the elastic properties of the cell membrane in the peripheral and central regions of the cell shows that the values of the local modulus of elasticity for different parts of the cell membrane differ significantly only at the penetration depth equal to 3.12 ± 0.029 nm. In general, it can be concluded that when experimenting within two hours after sample preparation with a probe with a large radius of curvature (more than 60 nm), the results of the force spectroscopy procedure do not depend on the probe insertion area.

To assess changes in the elasticity of cells depending on the loading time, a force spectroscopy procedure was performed at different speeds of movement of the sample table in the direction of the probe (300, 30, 15, and 1 nm/s). The indentation was performed in air, one hour after the preparation of the sample in the peripheral area of the cell at the selected speed, and the amount of bending of the console was recorded. Thus, the deformation of the sample at different speeds was ensured.

It was found that with a decrease in the loading rate of the erythrocyte membrane, the elastic resistance of the cell to mechanical deformation increases, i.e. the calculated value of the elastic modulus increases, respectively, the maximum

depth of the probe insertion that can be provided for the console with the selected stiffness decreases. Thus, the value of Young's modulus at the penetration depth of 5 nm at a sample displacement velocity equal to 1 nm/s is higher than at 30 nm/s and at 300 nm/s by 4.5 and 15.6 times, respectively. Thus, we can talk about the existence of a dependence of elastic properties on the rate of deformation of the cell, i.e. its viscoelastic behavior. The obtained results are consistent with the data of the study of the mechanical properties of individual erythrocytes using optical tweezers, according to which the cells exhibit viscoelastic properties under strong elastic deformation.

The results of the evaluation of the structural and mechanical properties of red blood cells indicate that for these cells, the issue of choosing the indentation area is not significant if the influence of the substrate is excluded during the experiment. And this is possible if the depth of the probe insertion does not exceed 10% of the cell thickness. In addition, there is a dependence of the modulus of elasticity on the depth of insertion of the probe, which can be explained by the heterogeneity of the properties of the objects under study, as well as by a certain influence of changes in the lateral tension of the membrane depending on the magnitude of deformation of the lipid bilayer.

Also, a direct relationship between the value of Young's modulus and the loading time has been experimentally established for red blood cells. However, the mechanism of this behavior of the erythrocyte membrane is not completely clear.

It should also be noted that studies of the mechanical properties of cells were carried out in the air, after drying on a substrate, which, of course, led to a decrease in the elasticity of their membranes, an increase in deformation resistance, and, as a consequence, an increase in the measured modulus of elasticity. In addition, it is required to use a contact deformation model more adequately, which would describe the experimental scheme of cell loading used and take into account its shell-type "construction". Therefore, the measured values of the elastic modulus should be considered as estimates, and the obtained dependences are more qualitative than quantitative.

Conclusion

The conducted studies reveal the methodological features of the application of atomic force microscopy, and in particular, the procedures of static force spectroscopy for assessing the local mechanical properties of the cell. The results of the studies indicate the importance of choosing the rate of contact action of the indenter on the surface of the erythrocyte membrane when assessing their elasticity. Based on the data obtained, it can be said that the estimated properties of the cell membrane largely depend on the ratio of the duration of relaxation transitions and the duration of temporary exposure during testing. The phenomena of the viscoelastic behavior of the cell require further study since the discovered mechanism of the direct dependence of the elastic modulus on the loading rate of the biological membrane is completely unclear.

The results of determining the mechanical properties of the erythrocyte membrane may differ significantly, which is caused by the lack of uniformity in the measurement conditions, and therefore makes it difficult to quantify and compare the results of studies. In order to obtain comparable results, it is necessary to strictly adhere to the methodological restrictions on the choice of experimental conditions and the mandatory indication of the methodology for determining the characteristics according to which they were calculated.

Thus, with strict regulation of the measurement conditions, AFM allows for a quantitative assessment of the mechanical properties of cells, which, in turn, opens up wide opportunities for the study of biological objects on a nanometer scale and the development of a new field of biomechanics – cellular elastography.

ACKNOWLEDGMENTS: The authors are thankful to colleagues of Saratov State Medical University named after Razumovsky for organizing the experiment.
CONFLICT OF INTEREST: None
FINANCIAL SUPPORT: None
ETHICS STATEMENT: The blood of individuals without any serious acute and chronic diseases was used for the research. All persons signed an agreement for volunteer participation in the experiment.

References

1. Nguyen-Tri P, Ghassemi P, Carriere P, Nanda S, Assadi AA, Nguyen DD. Recent Applications of Advanced Atomic Force Microscopy in Polymer Science: A Review. Polymers. 2020;12(5):1142. doi:10.3390/polym12051142
2. Xia F, Youcef-Toumi K. Review: Advanced Atomic Force Microscopy Modes for Biomedical Research. Biosensors (Basel). 2022;12(12):1116. doi:10.3390/bios12121116
3. An Y, Manuguri SS, Malmström J. Atomic Force Microscopy of Proteins. Methods Mol Biol. 2020;2073:247-85. doi:10.1007/978-1-4939-9869-2_14
4. Almalki GH, Rabah S, Said Arafa NM, Bahshwan SM. Immunohistochemical evaluation of the euphorbia inarticulata extract on liver and kidney tissues in hepatocellular carcinoma rats. Pharmacophore. 2022;13(2):33-40.
5. Allison DP, Mortensen NP, Sullivan CJ, Doktycz MJ. Atomic force microscopy of biological samples. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2010;2(6):618-34. doi:10.1002/wnan.104
6. Kodera N, Ando T. Visualization of intrinsically disordered proteins by high-speed atomic force microscopy. Curr Opin Struct Biol. 2022;72:260-6. doi:10.1016/j.sbi.2021.11.014
7. Dorobantu LS, Goss GG, Burrell RE. Atomic force microscopy: a nanoscopic view of microbial cell surfaces. Micron. 2012;43(12):1312-22. doi:10.1016/j.micron.2012.05.005
8. Gurkan UA. Biophysical and rheological biomarkers of red blood cell physiology and pathophysiology. Curr Opin Hematol. 2021;28(3):138-49. doi:10.1097/MOH.0000000000000639
9. Nobandegani AS, Motamedifar M. Antibiotic sensitivity profile of the bacterial isolates from the blood samples of the patients in different wards of a major referral hospital, Shiraz, Iran 2015-2016. Pharmacophores. 2019;10(2):30-6.
10. Alexy T, Dietrich J, Connes P, Toth K, Nader E, Kenyeres P, et al. Physical Properties of Blood and their Relationship to Clinical

Conditions. Front Physiol. 2022;13:906768. doi:10.3389/fphys.2022.906768
11. Baig BM, Abarian A, Baghaei S, Soroush S, Ataee Rad S, Pooromidi S, et al. Assessment of the Relationship between ABO Blood Group and Susceptibility, Severity, and Mortality Rates in COVID-19. Entomol Appl Sci Lett. 2021;8(2):32-6.
12. Pinho D, Carvalho V, Gonçalves IM, Teixeira S, Lima R. Visualization and Measurements of Blood Cells Flowing in Microfluidic Systems and Blood Rheology: A Personalized Medicine Perspective. J Pers Med. 2020;10(4):249. doi:10.3390/jpm10040249
13. Kim G, Lee M, Youn S, Lee E, Kwon D, Shin J, et al. Measurements of three-dimensional refractive index tomography and membrane deformability of live erythrocytes from Pelophylax nigromaculatus. Sci Rep. 2018;8(1):9192. doi:10.1038/s41598-018-25886-8
14. Vagabov IU, Kafarov ES, Zenin OK. Variants of Blood Supply to Kidney Segments According to 3D Anatomical Analysis. Entomol Appl Sci Lett. 2021;8(1):60-5.
15. Bhat SH, Ullah MF, Abu-Duhier FM. Anti-hemolytic activity and antioxidant studies of Caralluma quadrangula: potential for nutraceutical development in cancers and blood disorders. Int J Pharm Res Allied Sci. 2019;8(4):121-9.
16. Demchenkov EL, Nagdalian AA, Budkevich RO, Oboturova NP, Okolelova AI. Usage of atomic force microscopy for detection of the damaging effect of CdCl2 on red blood cell membrane. Ecotoxicol Environ Saf. 2021;208:111683. doi:10.1016/j.ecoenv.2020.111683
17. Chaudhuri O, Cooper-White J, Janmey PA, Mooney DJ, Shenoy VB. Effects of extracellular matrix viscoelasticity on cellular behavior. Nature. 2020;584(7822):535-46. doi:10.1038/s41586-020-2612-2
18. Assidi M, Dos Reis F, Ganghoffer JF. Equivalent mechanical properties of biological membranes from lattice homogenization. J Mech Behav Biomed Mater. 2011;4(8):1833-45. doi:10.1016/j.jmbbm.2011.05.040
19. Moroz VV, Chernysh AM, Kozlova EK, Sergunova VA, Gudkova OE, Khoroshilov SE, et al. Disorders in the Morphology and Nanostructure of Erythrocyte Membranes after Long-term Storage of Erythrocyte Suspension: Atomic Force Microscopy Study. Bull Exp Biol Med. 2015;159(3):406-10. doi:10.1007/s10517-015-2975-9
20. Himbert S, Blacker MJ, Kihm A, Pauli Q, Khondker A, Yang K, et al. Hybrid Erythrocyte Liposomes: Functionalized Red Blood Cell Membranes for Molecule Encapsulation. Adv Biosyst. 2020;4(3):e1900185. doi:10.1002/adbi.201900185
21. Parvini CH, Saadi MASR, Solares SD. Extracting viscoelastic material parameters using an atomic force microscope and static force spectroscopy. Beilstein J Nanotechnol. 2020;11:922-37. doi:10.3762/bjnano.11.77
22. Müller DJ, Dumitru AC, Lo Giudice C, Gaub HE, Hinterdorfer P, Hummer G, et al. Atomic Force Microscopy-Based Force Spectroscopy and Multiparametric Imaging of Biomolecular and Cellular Systems. Chem Rev. 2021;121(19):11701-25. doi:10.1021/acs.chemrev.0c00617
23. Tsukamoto S, Chiam KH, Asakawa T, Sawasaki K, Takesue N, Sakamoto N. Compressive forces driven by lateral actin fibers are key to the nuclear deformation under uniaxial cell-substrate stretching. Biochem Biophys Res Commun. 2022;597:37-43. doi:10.1016/j.bbrc.2022.01.107