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Erythrocyte surface modification due to the pharmacological treatment studied with atomic force microscopy

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The influence of some selected pharmacological compounds on the surface structure of human erythrocytes (red blood cells, RBCs) has been studied by means of atomic force microscopy (AFM). The imaging has been done both in the air environment on the fixed cells, and in a liquid (physiological conditions). RBCs are very sensitive to the osmotic changes in the solution, *e.g.*, increasing NaCl concentration in the solution to a value higher than 0.9% leads to the characteristic changes of the erythrocyte from a discoid-like shape to a very irregular one, the so-called "echinocyte", with a lot of ledges. Both contact and non-contact AFMs have been used to monitor the consecutive stages of RBC surface modification. Imaging the echinocytes immersed in isotonic solution has shown that the changes in RBC cells are fully reversible in the whole range of concentrations used in this investigation. Furthermore, the modification of the erythrocyte surface morphology induced by nifedipine, a drug used in the pharmacological treatment of hypertension, has been studied in this work.

1. Introduction

Since its discovery AFM [1] has been widely used to study biological and medical materials. The great advantage of AFM for biological sciences is that with this device one can image a fully developed geometrical structure of the surface of biological specimens both in air and in a liquid. This provides an opportunity for monitoring biochemical and physiological processes in real time at the molecular level or even with atomic resolution. High resolution studies are of particular importance, since conventional optical microscopy has its natural resolution limit due to diffraction. At first, the so-called "fixed cells" have been imaged with this technique [2]. More recently the number of successful applications of AFM to the imaging of cells is rapidly growing and the progress has been reported in the recent review by RATNESHWAR and SCOTT [3].

In normal conditions, human erythrocytes (red blood cells, RBCs) have a very characteristic discoid shape (and are sometimes called discocytes). That shape results from a wide variety of interactions between the cell and its environment, which are not always well understood. The discoid (toroidal) shape can be related to the role which the erythrocytes play in human organism. Such a shape has the higher surface to volume ratio than the spherical shape and can play an important role in the $CO_2 \leftrightarrow O_2$ exchange process. On the other hand, the smaller volume can allow the RBC for easy flowing through the capillary blood vessels. Different morphological factors such as different pH, high hydrostatic pressure, different substances can act on RBCs through molecular mechanisms that bring a number of distinguishable shapes. One of such deformations of the RBC discoid shape is the shape of the so-called "echinocyte". Such a cell has on its surface a set of ledges of different sizes.

The aim of the present study is to describe the pattern of RBC morphological changes, both due to the osmotic changes in the solution and due to nifedipine, a drug used in the pharmacological treatment of hypertension, belonging to the calcium channel blockers. Due to its hydrophobic characteristics, nifedipine is able to intercalate into membrane bilayers and therefore, it can induce morphological changes in RBCs as shown by GIRASOLE *et al.* [4].

2. Experimental

2.1. Experimental tools

Both contact AFM and non-contact AFM (also called DFM – dynamic force microscopy) [5], [6] measurements were carried out with a commercial instrument Thermomicroscopes CP equipped with a multimode head and a 100 μm scanner. Commercially available gold coated Si_3N_4 Park Scientific Instruments sharpened Microlevers, with a spring constant of 0.03 N/m and a pyramidal-shape sharpened tip with a nominal radius of curvature less than 20 nm, were used for contact mode imaging. For DFM, Park Scientific Instruments nc-Ultralevers, with resonant frequency of about 90 kHz, were used. For imaging in a liquid a commercial liquid cell (thermomicroscopes microcell) was used, which allowed us to control the amount of solution in the cell. Images of 256×256 points were collected for each scan with the scan rate maintained below 1 line/s.

2.2. Sample preparation

The blood samples were obtained from healthy human donors and they were investigated immediately after extraction. For imaging in ambient conditions a RBC sample was prepared without using any anticoagulating substances (like heparin or EDTA). The blood was dissolved with phosphate buffered saline (PBS) and then the cells were attached to the glass surface. That was achieved by spreading the solution manually on the glass microscope coverslips with surfaces covered by poly-L-lysine which can immobilise the cells and attach them to the glass surface. After 15 min the

RBCs attached to the coverslips were flushed with 2.5% glutaraldehyde solution for fixing the erythrocytes (fixing time 2 min). Subsequently, the glutaraldehyde solution was flushed off and the sample was dried. For imaging the cells in physiological conditions the procedure was similar but with no use of the glutaraldehyde solution and without drying the sample. Preparation of the cells treated with nifedipine was slightly different. Since nifedipine is poorly soluble in water, it was added to the investigated system as a homogenous emulsion prepared by sonicating the drug with 300 mOsm saline and 10 mOsm glucose kept in the ice water bath. The emulsions with different nifedipine concentrations were added to the RBCs prepared in the same way as for imaging in air. For each nifedipine concentration the images were collected for two samples: the one after an incubation time of 1 hour, and the other without incubation. All chemical substances (PBS, glutaraldehyde, nifedipine *etc.*) were purchased from Sigma Chemical Company, Poland.

3. Results and discussion

A typical toroid shape of human erythrocyte is shown in Fig. 1. For studying changes of the RBC morphology due to the osmotic changes in the solution, freshly extracted blood samples without the anticoagulating substances were diluted with the proper NaCl solution. Then the preparation procedure for air imaging was used. In Figure 2 one can see a set of the images of echinocytes obtained for four NaCl concentrations increasing from 1% to 3%. The images were collected using the contact mode AFM (constant force mode of 1.8 nN) for NaCl concentrations of 1% and 1.5%, respectively, and the non-contact mode (DFM) for NaCl concentrations of 2% and 3%. From the comparison of Figs. 2**a** and 2**b** with Figs. 2**c** and 2**d** one can conclude that in the images obtained in contact AFM the convolution of cell topography with the tip shape is clearly visible. For each concentration 3 samples were prepared with the incubation time of 1 min and 3 samples with the incubation time of 30 min. From our images one can

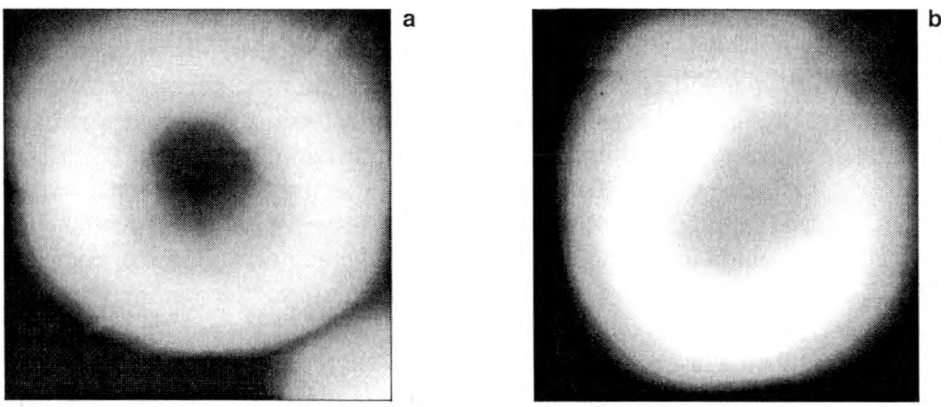

Fig. 1. AFM image of human erythrocyte with normal toroidal shape taken in air (**a**) and in physiological solution (**b**).

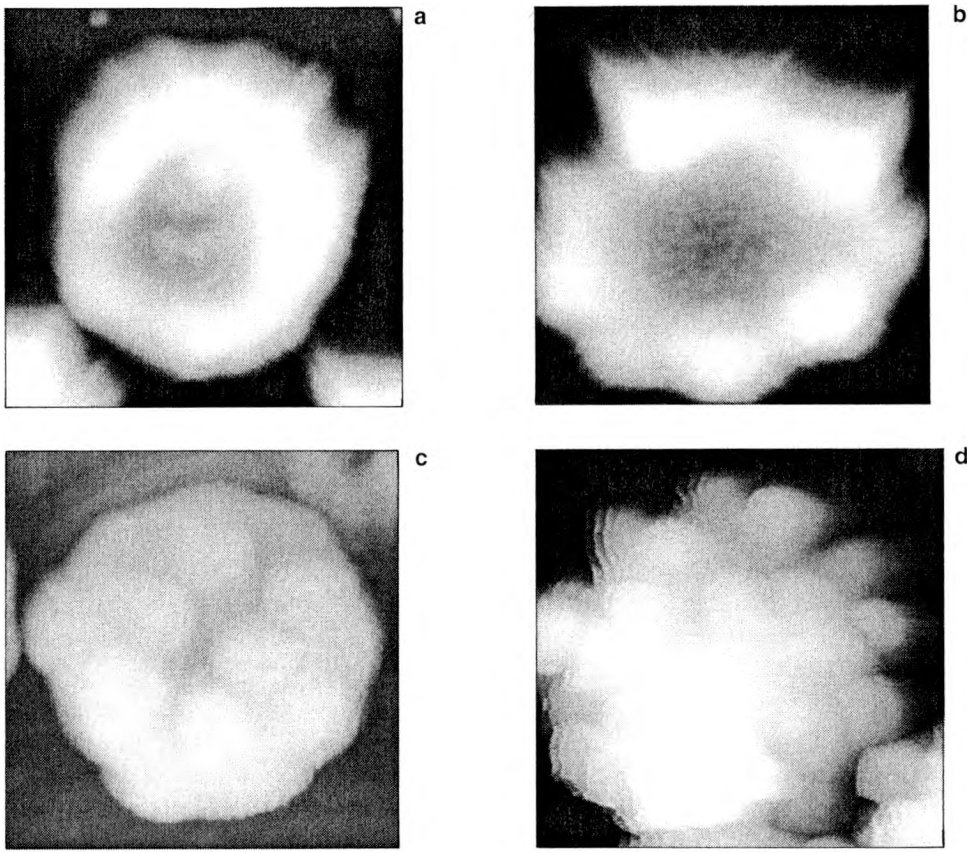

Fig. 2. Set of images of echinocytes for different NaCl concentrations. Contact AFM images for 1% (**a**) and 1.5 NaCl concentrations (**b**). DFM images for 2% (**c**) and 3% NaCl concentrations (**d**).

conclude that the changes in the RBC morphology were not homogenous on the whole sample, *i.e.*, not all the cells had changed their shapes. Therefore, a series of images was collected for analysis and the results are presented in Tab. 1. In this table both the average percentage of modified RBCs and the average results of the morphological parameters of modified RBCs for different NaCl concentrations are shown. In Figure 2**a** the first stage of erythrocyte shape modification is shown (1% NaCl concentration). It is seen that some small ledges were created on the cell membrane, however, the overall toroidal shape and the size of erythrocyte remained unchanged. On some cells the membrane modifications were visible only on one side of the cell. For the NaCl concentration of 1.5% (Fig. 2**b**) the changes in the membrane structure are more clearly visible. The sizes of ledges are bigger and they are visible on the whole cell surface but the overall shape of the cell is still preserved. For a still higher concentration (2%) more pronounced modifications are seen (see Fig. 2**c**) – the cell shape is less toroidal and more spherical, the average cell diameter decreases and ledges on the membrane are bigger. The number of modified RBCs increased to 80%.

T a b l e 1. Average morphological parameters of RBC modification for different NaCl concentrations.

NaCl concentration	Number of modified RBCs as a percent of total RBCs	Average diameter of modified RBC [μm]	Average height of the ledges [μm]	Average diameter of the ledges [μm]
1.0%	60%	8.0	0.10	0.1
1.5%	70%	8.0	0.25	1.0
2.0%	80%	7.5	0.30	1.0
3.0%	90%	6.5	0.38	1.0

T a b l e 2. Parameters of RBC modification at different nifedipine concentrations.

	Without incubation		1 h incubation time	
Nifedipine concentration	Average ledge height [nm]	Number of modified RBCs as a percent of total RBCs	Average ledge height [nm]	Number of modified RBCs as a percent of total RBCs
0.1%	45	48%	145	65%
0.2%	81	55%	146	70%
3.0%	52	45%	150	80%

For the highest studied in this work NaCl concentration (3%) the cells acquired a spherical shape with a diameter smaller than the one of native erythrocyte and a large number of densely packed ledges are seen on the whole membrane surface (Fig. 2**d**). In that case, all RBCs were modified and 90% of them had the shape shown in Fig. 2**d**.

The cells treated with nifedipine were imaged in the contact mode AFM. The results of the image analysis are collected in Tab. 2. It was found that the size of cell deformation was clearly dependent on the incubation time. At the first stage, without incubation, a certain amount of cells was deformed, but any dependence of the number of deformed cells and of the size of ledges on the nifedipine concentration was not observed. On the other hand, the size of the ledges was very similar to the one for 1 h incubation time and different nifedipine concentrations. The dependence of the number of modified RBCs on the nifedipine concentration was only seen for 1 hour incubation time. Similar results on the nifedipine-induced modification of the erythrocyte shape were presented by GIRASOLE *et al.* in [4]. The authors observed the dependence of RBC shape on the incubation time and a very strong dependence on nifedipine concentration was reported. They showed that the RBCs were completely destroyed after 1 h incubation time. The last finding was not confirmed by our study. Such different results could be explained by taking into account the differences in the applied method of cell preparation. Authors of paper [4] used cells not fixed with glutaraldehyde as it was done in the present work. Following the results of ZACHEE *et al.* [7] we know that erythrocytes, which are not fixed, changed substantially their shape during drying and they could even completely disappear. ZACHEE *et al.* [7] observed also that cells fixed with glutaraldehyde preserved their normal shape and size as confirmed by different techniques. In Figure 3 an example of AFM image of

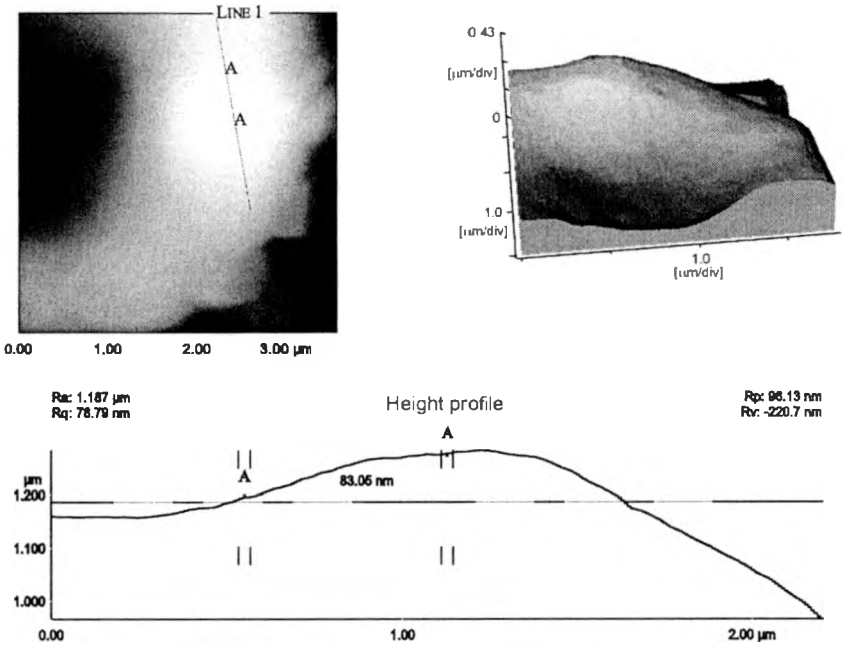

Fig. 3. AFM image of the RBC treated with 0.2% nifedipine without incubation. Images are presented together with cross-sections across the ledges and 3D visualisations.

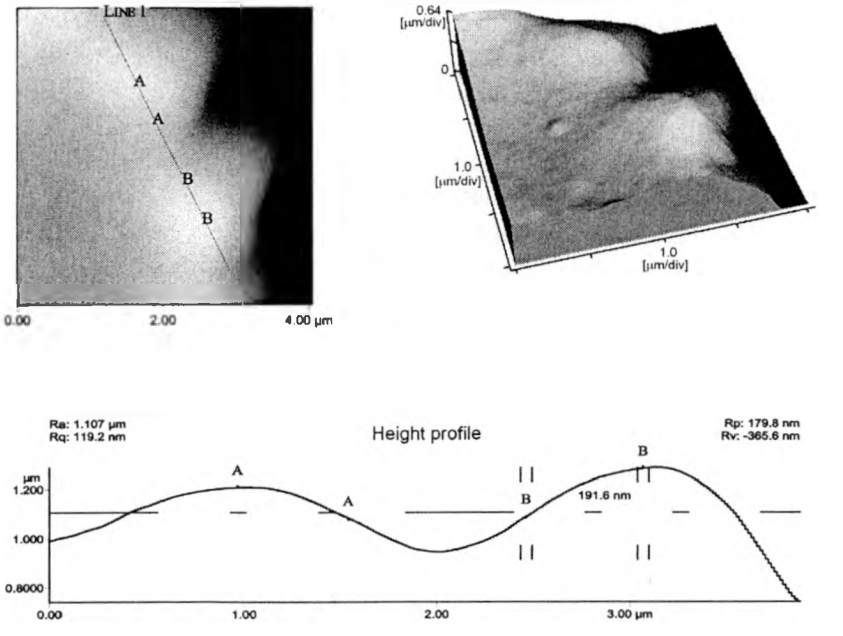

Fig. 4. AFM image of the RBC treated with 0.2% nifedipine after 1 h incubation. Images are presented together with cross-sections across the ledges and 3D visualisations.

RBC treated with 0.2% nifedipine without incubation is shown and in Fig. 4 the image of RBC treated with the same nifedipine concentration after 1 h incubation is presented. In both figures the scanned images with cross-sections and 3D visualisations are shown in order to visualise cell deformation changes during the incubation.

4. Conclusions

In this work we have demonstrated that different types of AFM imaging (contact and DFM, in air or in a liquid) could be effectively used in monitoring results of the erythrocyte surface modification due to pharmacological treatment. RBC morphological changes from discocyte to echinocyte due to osmotic changes in the solution known from the work of other authors were confirmed. We have found, however, that such changes are reversible within the investigated range of NaCl concentrations. The shape modification depends on the osmotic pressure of the solution but the incubation time has no influence on the size of deformations. On the other hand, the size of cell deformation for RBCs treated with nifedipine depends first of all on the incubation time. Consequently, in the first stage of the experiment with no incubation we have not found any dependence on the nifedipine concentration. After the incubation time of 1 h the sizes of ledges were largely independent on the nifedipine concentration but the number of modified RBCs was slightly growing with the concentration.

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