

Nanodiagnostics performed on human red blood cells with atomic force microscopy

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Atomic force microscopy can yield valuable information concerning basic physical properties as well as alterations of human red blood cells. Erythropoietin is a hormone that is naturally produced in the kidney to stimulate the growth of red blood cells. Administration of genetically engineered synthetic erythropoietin stimulates the production of additional red blood cells. Therefore erythropoietin is used for blood doping in serious sports. The present study aims at investigating any differences in structure and stiffness of red blood cells which are produced body own or with synthetic erythropoietin. The samples are prepared via standard methods, and atomic force spectroscopy with trigger forces of three micronewtons is performed in ambient air. The penetration depth does not reveal statistically relevant differences in the two types of red blood cells. Furthermore, cells with a penetration depth four times as large as healthy ones are encountered in the samples of one donor. Subsequent medical examination revealed a rare type of diabetes. Atomic force spectroscopy shall serve as fast screening method for nanodiagnostics of diseases that alter surface nanomechanical properties.

Keywords: Atomic force microscope, Erythrocytes, Red blood cells, Erythropoietin, EPO, Force mapping

Introduction

With the atomic force microscope (AFM) it is possible to mechanically investigate the soft surface of erythrocytes. The AFM provides insight into topography as well as nanomechanical properties (such as stiffness) of the human red blood cells as well as the investigation of tip induced deformation of the cells. In force mapping mode it is possible to mark points of interest on the cell. With a certain preset force the cantilever tip can be pressed into the marked points and thereby probe local parameters at unprecedented resolution.

Cross *et al.* report in 2007 in Nature Nanotechnology a change in stiffness in cancer cells as compared to benign cells:¹ samples of lung, breast and pancreas cancer were studied with AFM. It was found that the stiffness of metastatic cancer cells is more than 70% softer with a standard deviation over five times narrower than the benign cells that line the body cavity.

Dulinska and co-workers investigated erythrocytes from patients with haemolytic anemias and patients with anisocytosis and found statistically relevant differences in stiffness.² Inspired by the abuse of erythropoietin (EPO) in serious sports (doping) it is investigated if there is any difference in the structure and stiffness of red blood cells which are produced due to synthetic EPO and body own EPO.

For the experiments the stiffness of blood samples of renal insufficient patients (i.e. patients with kidney problems), who are medicated with synthetic EPO and blood samples, of a control group (healthy individuals) are compared. The maximum age of donors was 50 years.

Bioimaging with the AFM has become an ambitious field of research.^{3,4} There are several reasons for this development; the most important is that the AFM is a general purpose instrument for analysing surfaces at ultrahigh resolution, in ambient, fluid or vacuum conditions. Some of the applications are analysis of DNA and RNA, protein–nucleic acid complexes, chromosomes, cellular membranes, proteins and peptides, molecular crystals, polymers and several other biomaterials.^{3,4} Compared to other analytic instruments the AFM and especially the ambient AFM has a variety of advantages. The main advantage is the ability to analyse non-conducting samples without additional preparation such as metallising with gold or similar techniques.

Techniques of AFM can also be used for manipulating vegetative samples and measuring mechanical properties of cell membranes.⁵

Overview of erythrocytes and erythropoietin

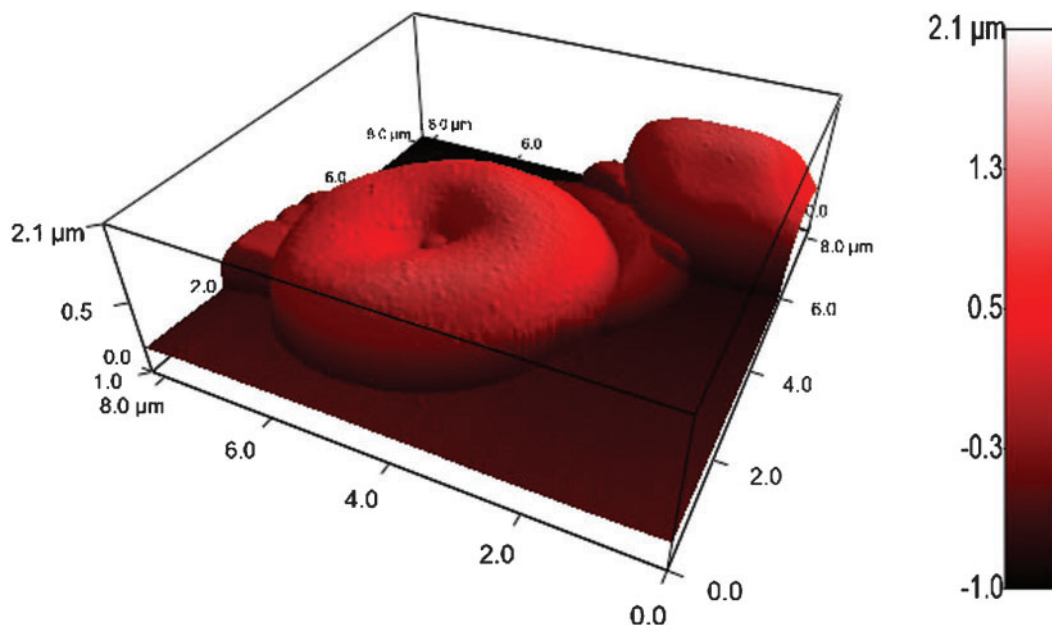
The erythrocytes (Fig. 1) give the blood its red colour. Blood contains different types of cells and the so called blood plasma (a fluid rich of proteins such as the coagulation factors). Red blood cells (erythrocytes), white blood cells (leucocytes) and platelets (thrombocytes) make the cellular part of the blood.

The red blood cells carry oxygen to the periphery of the body and carbon dioxide from there back to the lung

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1 Image (AFM) of red blood cells in air (three-dimensional height image, scan size $8 \times 8 \mu\text{m}^2$)

to exchange the gases and start the circle again. This is possible with the help of haemoglobin. It makes $\sim 30\%$ of the whole red blood cell. It is responsible for the adequate transport, because the CO_2 is transported in H^+ and HCO_3^- .⁶

This whole system is a major part of the acid/base management of the body.

There are about 4.5–5 million red blood cells per μL blood.

Some facts about red blood cells are:⁷ diameter, $7.5 \mu\text{m}$; thickness, $2 \mu\text{m}$; shape, biconcave; surface area, $135 \mu\text{m}^2$; volume, 90 femtolitres.

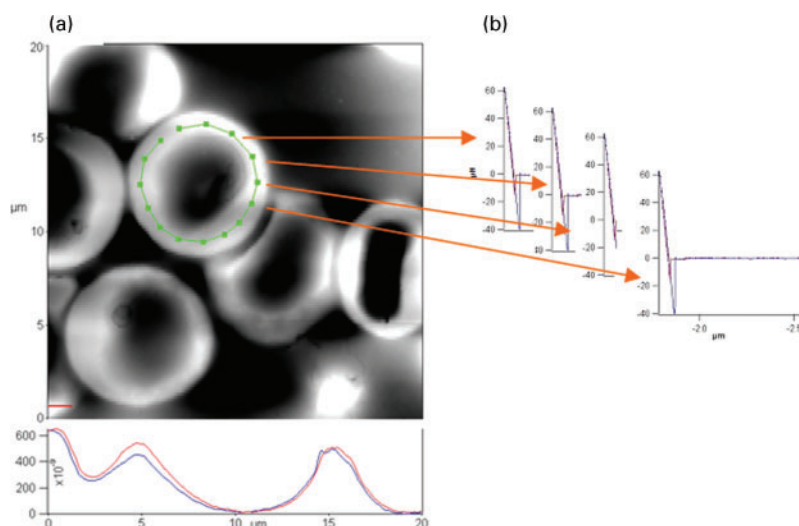
Erythropoietin is a hormone which is produced by the kidney. It stimulates the bone marrow to produce red blood cells. Synthetic EPO is clinically used for treating renal caused anemia and kidney diseases.⁶ In serious sports recombinant erythropoietin is used to increase the number of red blood cells. It is a glycoprotein that is generally expressed in Chinese hamster ovary cells

transfected with DNA encoding for human erythropoietin. It enhances the number of erythrocytes by about 5–20%. This leads to a higher oxygen transfer and therefore athletes achieve better performance. Synthetic EPO is difficult to detect, because it is metabolised within 6–12h and is very similar to the naturally occurring respective hormone.

A medical device based on a method similar to AFM stiffness evaluation that fast and reliably detects doping with EPO directly on site would find wide applications in serious sports.

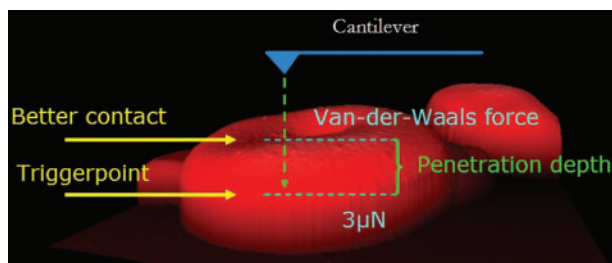
Materials and methods

Red blood cells of untreated patients and of patients treated with erythropoietin and its synthetic equivalent EPO are investigated. The kidneys produce the erythropoietin in the interstitial cells. A dysfunction of the kidney causes a decreased production of erythropoietin.



a cells are imaged and a path with 15 points is set; b on these 15 points, force curves are recorded

2 Force curve acquisition



3 Penetration depth evaluation: The penetration depth is the distance between better contact, i.e. the cantilever position when the cell is touched, and the cantilever position when the trigger point of 3 µN is reached

Such patients get EPO as medication to antagonise a renal caused anemia. After the standard procedure of preparation⁸⁻¹¹ of the blood samples the cells are imaged with the AFM using dynamic mode. Subsequently AFM force curves are recorded. The force curve parameters of the healthy and with EPO treated blood samples are finally compared.

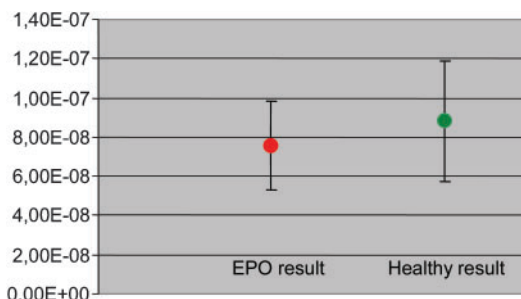
Sample preparation

The blood obtained from renal insufficient patients and a control group from the department of nephrology and kidney dialysis of the AKH, anticoagulated with heparin, is attached to glass slides. This is achieved using poly-L-lysine solution (0.1% in water), glutaraldehyde (1% solution) and phosphate buffer saline (PBS). The glass slides are dipped in the poly-L-lysine solution, rinsed with glutaraldehyde and washed with the PBS. The blood is diluted in PBS 20 times and attached to the chemically treated glass slide surface. After 5 min the glass slides are treated with the glutaraldehyde again. After interacting for 1 min the blood cells are washed with PBS and the sample is mounted on the AFM.⁸⁻¹¹

Atomic force microscope

The AFM used in the present study is the MFP-3D from Asylum research in Santa Barbara, CA, USA. Main parts of the AFM are the cantilever with a sharp tip at its end, which is used to scan the sample surface, an infrared laser, which is aligned with the cantilever, and a deflection sensor, which is a photodiode detecting the position of the cantilever. The measuring principle is based on the fact that there are very small forces (<1 nN) between the AFM tip and the sample surface.

The images are recorded in dynamic mode to prevent damaging the sample by scratching over it, with cantilevers with 70 kHz resonance frequency in air



4 Penetration depth (m) for EPO cells and healthy cells

and a spring constant of 1.8 nN nm⁻¹ (Olympus OMCL-AC240TS). The scan frequency is 0.43 Hz (i.e. a little bit less than two lines per second), the image size is originally 20 × 20 µm (Fig. 2a), regions of interest are subsequently scanned with smaller scan size.

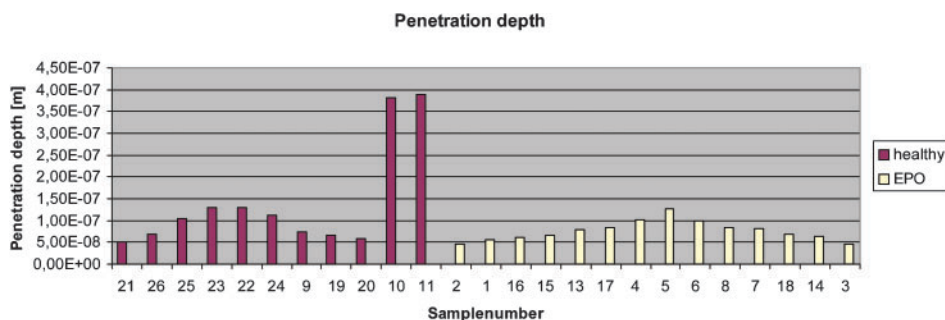
After imaging the erythrocytes, force spectroscopy is performed on each single cell along preset paths. A path with 15 points is created (Fig. 2a) and the trigger point of the force is set to 3 µN. Then, force v. separation curves are recorded (Fig. 2b).

About 200 force v. separation curves are recorded per sample, 25 samples from 24 individuals are investigated. In some of the curves the first curve is no real force curve because the tip sample distance is often too large to reach the sample and with it the preset trigger point. In these cases the respective first curves are excluded from analysis. To calibrate the system a force curve is performed on a hard glass surface and the deflection inverse optical lever sensitivity is set accordingly. At the edges of the cells small artefacts due to the feed back mechanism of the system are obtained.

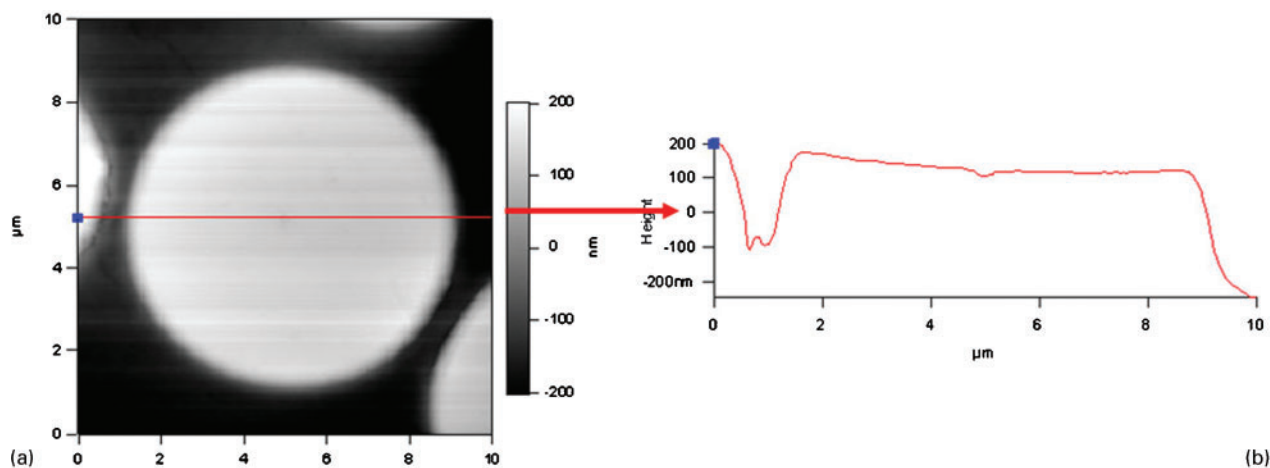
For the question if there is a difference in the stiffness and plastic deformability of healthy and EPO medicated patients, the most significant parameter is the penetration depth. The penetration depth is a parameter for the deformability of the cells. It is evaluated by the position of the maximal movement of the piezo sensor in z direction, which is the coordinate when the sensor reaches the trigger point of 3 µN, minus the position of the sensor when the tip is in first contact with the surface of the red blood cell (better contact, Fig. 3).

Results

Twenty-six blood samples of 14 EPO patients and 10 people from the control group with acquisition of about 200 force v. separation curves per sample were investigated.



5 Penetration depth of all samples



a height trace; b height profile along line in image
 6 Abnormal red blood cell (scan size $10 \times 10 \mu\text{m}^2$)

The penetration depth shows no statistically significant difference in healthy and EPO blood samples (Fig. 4).

However, the penetration depth of samples 10 and 11 is four times higher than the penetration depth of the other samples (Fig. 5).

There are also abnormalities of the surface of these blood cells (Fig. 6). The erythrocytes do not have the typical donut shaped form. These blood cells are oblate and very flat (Fig. 6). These two blood donations are from the same donor. The measurements are repeated in order to ensure that no destruction of the sample during transportation or preparation took place. The result is the same. After more detailed medical investigation a rare case of diabetes is diagnosed in this donor.

Discussion, conclusion and outlook

The blood donations of healthy patients in comparison with the blood donations of EPO treated patients show no statistically relevant difference regarding the penetration depth obtained at a given trigger force. Subsequent investigation concerning further parameters of the cell membrane of red blood cells such as viscosity might yield a fast and reliable method to detect doping directly on site in serious sports.

The blood donations of healthy patients in comparison with the blood donation of a person with a rare case of diabetes shows a statistically relevant difference regarding the penetration depth obtained at a given trigger force: the penetration depth is larger for the blood cells of the diabetic, indicating softer cells. The AFM successfully proves as a nanodiagnostic tool. Minamitani and co-workers measured the deformability and viscoelasticity of erythrocytes of patients with diabetes mellitus, the common type of diabetes, by microchannel flow systems and AFM. The blood cells of the patients with diabetes mellitus are harder than the control group.^{12,13} The reason for the softening of erythrocytes in the rare case of diabetes and the hardening of erythrocytes in diabetes mellitus is yet to be determined.

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