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Investigation of the swelling of human skin cells in liquid media by tapping mode scanning force microscopy

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Abstract. The swelling of individual human skin cells (so-called corneocytes) was studied in distilled water and hexadecene, respectively, by tapping mode scanning force microscopy. The area, the mean height, and the volume were recorded and compared to data of the same cell, which has been obtained under ambient conditions. In distilled water, we found an average swelling in height and volume of about $(50 \pm 10)\%$, but no significant increase of the area. Additionally, phase-contrast images suggest a significant change of the viscoelastic properties of the corneocytes. On the other hand, the corneocytes behaved inertly when exposed to hexadecene as an example of a non-polar solvent.

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The skin plays an important role for the regulation of the water loss of terrestrial animals; it was this specific barrier property which enabled life to explore land as habitat during evolution. The water-regulation mechanism is still crucial for survival for any terrestrial: damage to skin such as, e.g., large burns always poses a great danger to life due to the uncontrolled loss of water. Responsible for this permeability barrier is the uppermost layer of the skin, the stratum corneum, despite its thickness of only ≈ 5–$10\,\mu$m. It is a highly orientated layered structure consisting of alternating cornified cells (the so-called corneocytes) separated by multilamellar lipid layers [1].

The individual cornified cells, which form these layers, are continuously produced in a deeper layer of the skin, the stratum basale. During their differentiation from the stratum basale to the stratum corneum, the cells synthesize proteins and lipids, die, and transform into the comparatively hard corneocytes of typically 30–40 μm diameter, with only 200–300 nm thickness. These corneocytes can easily be imaged in a scanning force microscope. Their comparatively stiff structure originates from an internal network of keratin, a sulfur-containing fibrous protein, which is embedded in a protein matrix and surrounded by a rigid cornified envelope [2, 3]. Keratin has an affinity to water [4], which acts as its plasticizer. Thus, in order to gain elasticity, the corneocytes need to be hydrated. Hydratization, on the other hand, causes an increase in volume. This swelling of the corneocytes is directly correlated to the regulation of the transcutaneous water loss: it enhances the pressure on the intermediate lipid layers, which thus undergo a phase transition to a state less permeable to water.

A first quantitative study on the swelling of corneocytes was performed by Norlén et al. [5] by means of confocal laser-scanning microscopy. Restricted by the limited resolution of this method ($\approx 0.5\,\mu$m in the vertical dimension [6]), individual corneocytes were not investigated, but thin cuts of about 5 mm × 5 mm with an average thickness of $(17.2 \pm 3.6)\,\mu$m (number of samples $n = 8$), which naturally also included the intermediate lipid layers. The average thickness increase between hydrated and dry stratum corneum pieces was found to be $(3.6 \pm 2.2)\,\mu$m, corresponding to an average relative increase of $(26.3 \pm 16.3)\%$, which was well within the resolution limit of the microscope used. In the present study, however, a different approach was chosen: by comparison of the dimensions of individual corneocytes, which are deposited on a silicon substrate and subsequently imaged by tapping mode scanning force microscopy with and without liquid media, the swelling of single corneocytes was investigated directly and without associated lipids.

1 Experimental

The corneocytes examined in this study were washed away from the inside of the forehand, close to the wrist, by a non-ionic detergent consisting of 0.3% Triton X100 [7] in distilled water. The amphiphilic Triton dissolves the lipids between the corneocytes, thus destroying the cohesion of the upper layers of the stratum corneum. As a consequence, individual corneocytes segregate from the topmost layers and can easily be sucked up with a pipette in a drop of liquid.

As a substrate, small parts of a silicon wafer were used, since this material is very flat over a large distance, com-

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paratively hard, and (semi)conductive, which reduces electrostatic problems. To make sure that the corneocytes are attached strongly enough to the silicon, it was silanized with N-(2-aminoethyl)-3-aminopropyltrimethoxysilane [8] in order to establish chemical bonds between the substrate and the corneocytes after sample deposition. We followed the preparation guidelines given in [9]; without this procedure, the corneocytes did not stick to the substrate during measurements under liquids. After silanization, a few drops of the cell suspension were deposited onto the substrate, which was subsequently allowed to dry overnight. Finally, the sample surface was rinsed with distilled water in order to remove both unbound corneocytes as well as all excess of Triton X100.

The experiments were carried out with a Nanoscope IIIa scanning force microscope [10] operated in tapping mode in order to minimize the deformation of the corneocytes due to normal as well as lateral tip–sample forces. This is especially important while measuring in distilled water, since the swollen corneocytes turned out to be considerably softer than they are in the non-hydrated state (see Sect. 2). Imaging was performed with silicon cantilevers, which possessed resonance frequencies of ≈ 60 kHz and spring constants of 1–5 N/m, at typical scan rates of 0.3 Hz per line; topography and phase were recorded simultaneously. Comparative measurements in air and in liquid were always carried out with the same cantilever, using the Nanoscope's liquid-cell equipment.

2 Results and discussion

A typical image of a corneocyte recorded under ambient conditions is presented in Fig. 1a. In this micrograph, as well as in Fig. 2, bright colors indicate high and dark colors low

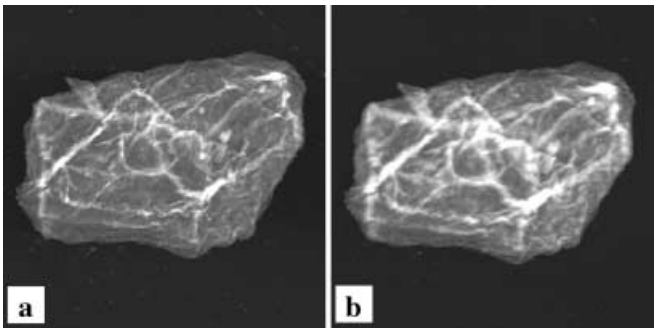

Fig. 2a,b. Force micrographs of a corneocyte recorded under ambient conditions (**a**) and in distilled water (**b**). The mean height increases from (211 ± 3) nm in the non-hydrated state to (330 ± 6) nm in water [11]. Scan size in both images was 47 μm × 47 μm; the maximum height corresponds to 1.2 μm

parts of the surface. All corneocytes investigated were easily distinguishable from the very flat silicon substrate and showed a very rough top surface (cf. the cross section displayed in Fig. 1b). This intrinsic roughness is presumably due to the internal 'skeleton' of the cell formed by keratin and the cell–cell contact remnants. Frequently, bright 'ridges' running all over the cell's surface, as is observed, e.g., in Fig. 2, are found. Here, Fig. 2a was measured under ambient conditions, whereas the data presented in Fig. 2b were acquired in distilled water. Since the grey scale which represents the local height is identically chosen for both images, we can easily note an increase in the mean height of the corneocyte, which changes from (211 ± 3) nm under ambient conditions to (330 ± 6) nm in water [11]. In contrast, the lateral dimensions seem to remain unchanged.

This issue is further elaborated in Fig. 3, which displays the histograms of the local heights. Both histograms feature

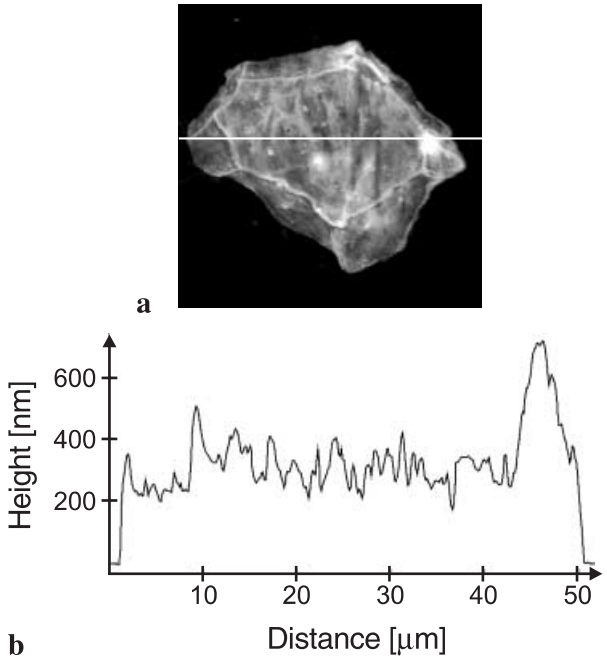

Fig. 1. **a** Image of a human corneocyte with a mean height of (329 ± 2) nm [11] acquired in air using tapping mode scanning force microscopy; the scan size was 55 μm × 55 μm. **b** Cross section taken along the line indicated in **a**, demonstrating the high surface roughness of the corneocyte

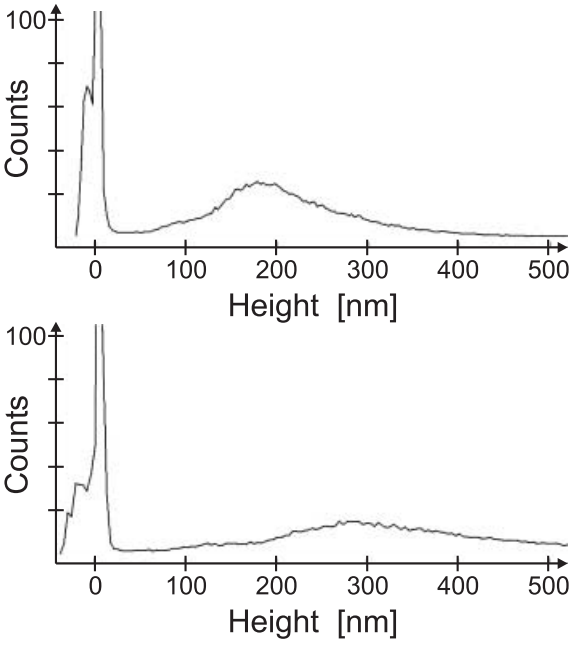

Fig. 3a,b. Histograms of the local heights as recorded in the measurement displayed in Fig. 2: **a** ambient air and **b** distilled water. The larger distance between the two peaks in **b** compared with **a** illustrates the increase of the cell's mean height in water. For a critical discussion see [12]

Table 1. Volume, area, and mean height measured for four different corneocytes in air and distilled water [11]. Additionally, the proportional change in water compared to the measurements under ambient conditions is given in per cent. In all cases, no changes of the dimensions could be recorded with time. This indicates that the swelling process takes place on a time scale faster than about 7 min, which represents the shortest time difference between the acquisition of a data set in air and the acquisition of a corresponding data set in distilled water

#	Mean height			Area			Volume		
	Air [nm]	Dist. water [nm]	%	Air [μm^2]	Dist. water [μm^2]	%	Air [μm^3]	Dist. water [μm^3]	%
1	341 ± 8	506 ± 5	48 ± 4	830 ± 25	802 ± 11	-3 ± 3	283 ± 11	406 ± 7	43 ± 6
2	211 ± 3	330 ± 6	56 ± 4	982 ± 19	967 ± 17	-2 ± 3	207 ± 5	319 ± 8	54 ± 5
3	213 ± 4	345 ± 2	62 ± 3	1055 ± 24	1050 ± 6	-1 ± 2	225 ± 7	362 ± 3	61 ± 5
4	335 ± 6	480 ± 4	43 ± 3	1112 ± 24	1130 ± 30	2 ± 4	373 ± 10	542 ± 15	46 ± 6

two distinctive peaks: a sharp, narrow peak characterizing the silanized silicon substrate and a broad peak, which corresponds to the surface of the corneocyte. As an effect of the water, we note a significant broadening of the peak which belongs to the corneocyte; additionally, the distance to the substrate peak increases, indicating the increase of the mean height [12].

Table 1 summarizes our experimental data regarding the swelling in distilled water. As the most important result, we find in water an increase of the volume and the mean height of about $(50 \pm 10)\%$, whereas no significant lateral swelling (i.e., a change of the substrate area which is covered by the cell) can be observed. This data can be compared to those from Norlén et al. [5], who recorded a height increase of $(26.3 \pm 16.3)\%$ and an area increase of $(8.4 \pm 1.4)\%$. There are several possible reasons for the discrepancy to our results. (1) In the present work, we were able to study individual corneocytes, which were free from associated lipids. Since they are not part of an intact piece of the stratum corneum, they also do not feel any external pressure caused by the swelling of neighboring corneocytes, which might limit their expansion. (2) Corneocytes from deeper layers of the stratum corneum might swell differently due to their less-condensed structure than corneocytes from the uppermost layers, which were investigated in the present study. (3) The stratum corneum investigated by Norlén et al. was extracted from the breast region. Our samples, however, were taken near the inside of the wrist. (4) The samples prepared by Norlén et al. showed thicknesses which were above typical values for the stratum corneum. This either points to a structural modification of the stratum corneum caused by their sample preparation, or it indicates that the samples included some lower-lying skin layers, despite the fact that the authors tried to remove all residual layers from such samples [5]. (5) Finally, statistics might also play a role: Norlén et al. studied samples of 20 different females (area increase) and 8 different females (height increase), respectively, of 16–49 years of age, whereas our samples were taken from one male test person (26 years old).

On the other hand, mainly two different explanations might account for the absence of any change of the lateral dimensions. First, it could be an artefact of the silanization. Keeping in mind that we use silanization to firmly attach the corneocytes to the substrate, we might assume that this procedure also prevents any lateral movement. And second, it could represent an intrinsic feature of isolated corneocytes, as we speculate below.

The hydrophilic keratin network inside the corneocytes, which acts as a kind of internal reinforcement stabilizing the shape of the corneocyte, is known to be well-orientated. X-ray diffraction studies have shown that the keratin fibrils are essentially oriented in the plane of the corneocyte [13]. The specific arrangement of the keratin fibrils reduces the ability of the cell for lateral expansion, since the keratin fibrils can withstand quite high stress without elongating. This is an important feature for the functionality of the skin, which should not increase their surface area considerably if it is in contact with water. In the vertical direction, however, such restrictions are minimal, and it has been argued that the swelling in this direction might only be limited by the elasticity of the corneocyte envelope [5, 14], which surprisingly allows a volume change of up to 50%.

Additional information on the nature of corneocyte swelling can be obtained by the analysis of phase images. In Fig. 4, the phase images are displayed which were simultaneously recorded with the topographical images presented in Fig. 2. At ambient conditions (Fig. 4a), the phase image shows no difference between the silicon substrate and the cell, while a striking contrast is observed in water (Fig. 4b). In recent years, it has been found that phase contrast in tapping mode can be associated with tip–sample interactions that involve energy dissipation such as adhesion-energy hysteresis and viscoelasticity [15–18]. Thus, Fig. 4a implies that both the adhesion and the viscoelastic properties of the corneocyte and the silicon substrate are roughly identical. This sounds plausible since the non-hydrated corneocytes are known to be hard and brittle, as already discussed above. On the other hand, we tentatively assign the considerable phase contrast observed in Fig. 4b to a significant change of the viscoelastic

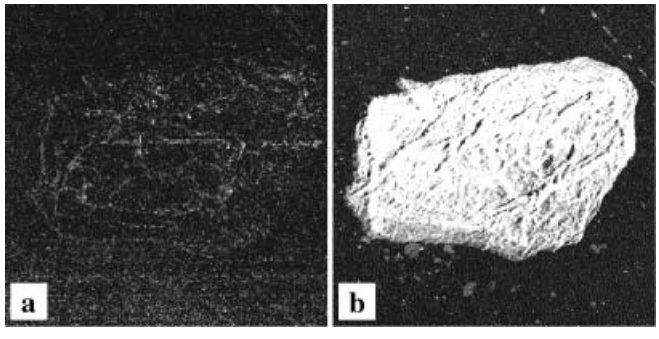

Fig. 4a,b. Phase images of a corneocyte acquired under ambient conditions (**a**) and in distilled water (**b**). The images were recorded simultaneously with the topographical micrographs displayed in Fig. 2. Scan size in both images was $47\ \mu m \times 47\ \mu m$; the full grey scale from dark to white corresponds to a maximal phase difference of 30°

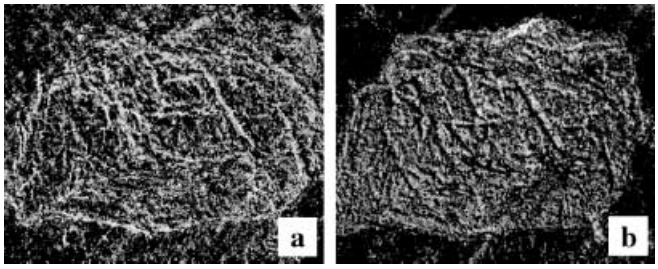

Fig. 5a,b. Phase images of a corneocyte obtained under ambient conditions (**a**) and in hexadecene (**b**). No significant phase contrast can be observed. Scan size: 44 μm × 36 μm; the full grey scale includes a maximal phase difference of ∼ 20°

properties of the hydrated corneocyte due to swelling, since we do not expect a different local viscoelasticity of the silicon substrate under liquids compared with the non-wetted case.

Finally, we also performed a series of five independent measurements in hexadecene, a non-polar solvent. The above-mentioned specific affinity of keratin for water suggests that we might not observe any conformational change due to swelling in a non-polar medium. Our experimental results in hexadecene indeed confirm this expectation: neither the mean height nor the area (and thus the volume) changed within the statistical error margins. Furthermore, we could also not detect any significant phase contrast (see Fig. 5), which has been identified in the former paragraph as an indication for corneocyte swelling.

3 Conclusions

In this work, we used tapping mode scanning force microscopy to analyze the shape of individual corneocytes before and after swelling in distilled water. Statistical analysis revealed an increase in the mean height and the volume of about $(50 \pm 10)\%$, but we did not record significant changes in the lateral dimensions. These findings correspond to our observation of a striking contrast in phase images after swelling, which is tentatively interpreted to express a change of the cell's viscoelastic properties. In contrast, measurements performed in hexadecene could not demonstrate any swelling, nor did we find indications suggesting a change of the material properties of the corneocytes.

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11. We would like to point out that the error given here describes only the statistical error assuming a correct calibration of the microscope. This error, which is very low due to averaging, is the important quantity if the proportional changes between two measurements acquired using the same calibration should be investigated. However, the precision of the calibration itself (representing a systematic error) is only ±10% and has to be additionally considered if absolute values are of interest
12. In this context, two additional issues should be noted: (1) the mean heights of (211 ± 3)nm (non-hydrated state) and (330 ± 6)nm (distilled water) are not identical with the most probable heights of ≈ 180 nm (a) and ≈ 285 nm (b), as can be derived from the histograms, since the mean heights have to be interpreted as the difference between the centers of mass of the two different peaks. The peaks which belong to the corneocytes, however, are very asymmetric due to long tails to high local heights (the maximum height recorded in Fig. 2b is 1.2 μm), explaining the values for the mean heights given above. (2) The width of the peaks represents the variation of the actual height on the surface of the corneocyte, and is thus naturally much larger than the error of the mean height, which is in the nanometer range due to statistical averaging [11]
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