Atomic Force Microscopic Studies on Erythrocytes From an Evolutionary Perspective

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ABSTRACT

We examined atomic force microscopy (AFM) and lateral force microscopy (LFM) images of human, avian, reptilian, amphibian, and piscine erythrocytes to determine whether the general pattern of erythrocyte membrane architecture has been largely conserved in the course of phylogenetic evolution or relatively minor modifications have taken place. The general pattern of the cell surface structure is indeed very similar among the phyla examined. The surface features include a number of blebs or globular structures and hole-like depressions. Such features are particularly clear in fish (Heteropneustes sp.), in which globular blebs are arranged in tiers around the depressions. The same pattern is found in the other phyla, although the sizes of the blebs and depressions vary. The depressions are ~340 and ~100 nm in diameter in chickens and fish, respectively, and are smaller in other phyla. The images of human erythrocytes presented here show holes more clearly than the images obtained by Zhang et al. (Scanning Electron Microsc., 1995; 9:981–989), who showed for the first time the highly uneven surface of these cells. The globules range in size from ~50–150 nm in diameter. These nanostructures have a width of approximately 333–1,000 atoms, assuming that the average dimension of an atom is 1.5 Å. The size range of the holes is approximately 40–432 nm (equivalent to a width of approximately 266–2880 atoms). LFM images, which take into account the lateral component of the force, represent the variation of surface friction (roughness) on the erythrocyte surface. This is very clear in the toad images, which show well-ordered strata that have not been revealed in ordinary AFM images.

Key words: AFM; LFM; erythrocytes; evolution; nanostructures

The atomic force microscopy (AFM) technique has been applied in the last decade on various biological samples (Bustamente and Keller, 1995). This revolutionary method requires no sample preparation at all (being based on the interaction of sample surface atoms with the probe atoms), and can obtain images at significantly higher magnifications compared to scanning electron microscopy (SEM). This technique can therefore reveal hitherto unknown structures of the cell surface.

In previous studies, human erythrocytes were imaged with AFM (Zhang et al., 1995; Ohta et al., 2002). These studies revealed bleb-like structures on the erythrocyte surface. Sporadic attempts to image erythrocytes from other vertebrates have also been made (Albrecht, personal communication). In this study we describe a general pattern of erythrocyte membrane structure in five phyla, which appears to have been conserved in the course of vertebrate evolution. We also present lateral force microscopy (LFM) images of some of the samples. An LFM image is obtained by taking into account the horizontal component of the force. This reveals the variable frictional force

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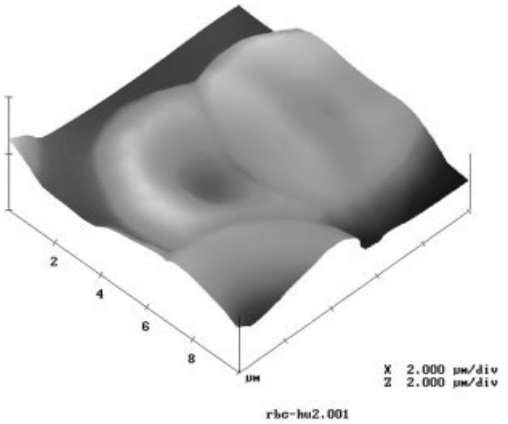

Fig. 1. AFM image of a human erythrocyte showing the normal structure following air drying.

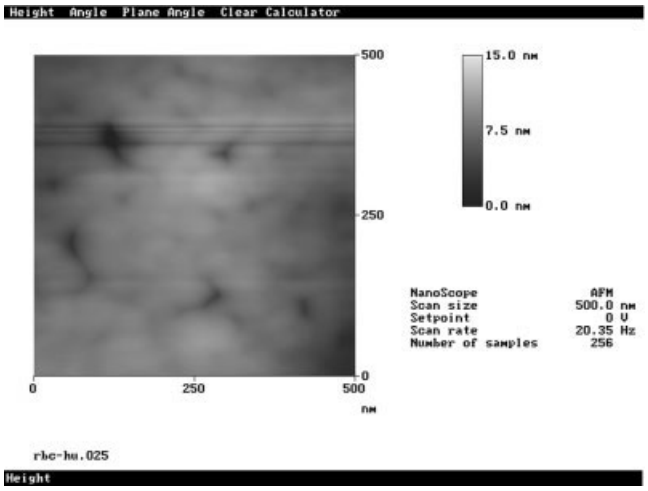

Fig. 2. AFM image of a human erythrocyte showing blebs (globules) and holes.

(and therefore the roughness) on the surface of a human erythrocyte (Guha et al., 2002).

MATERIALS AND METHODS

Erythrocytes were obtained by whole-blood smears on glass slides. The samples were cut out in 8 × 8 mm pieces. At first, we used low magnifications of AFM to determine whether the overall shape of the erythrocyte was maintained, and then we used progressively higher magnifications. All of the AFM images were taken in the contact mode in air. A Digital Instruments nanoscope (Inter-University Consortium, Indore, India), E series scanning probe microscope (ESPM) system, with a 100-μm, triangular-type cantilever attached with a silicon nitride probe (800 nm thick) was used. In this manner, a comparative study of erythrocytes from five phyla was performed. We acquired LFM images of some of the samples using the LFM mode in the same instrument.

RESULTS

AFM Images

Human erythrocyte. Figure 1, at low magnification (10 μm × 10 μm), shows the normal shape of whole erythrocytes. Their appearance indicates that drying during processing resulted in little or no structural distortion. At higher magnification (Fig. 2), a 500 nm × 500 nm area shows holes or depressions arranged between multiple surface blebs. Measurements of these features, according to the scale adjacent to Figure 2, are presented in Table 1. In another sample (image not shown), the diameters of five holes are 55, 86, 75, 75, and 90 nm, respectively.

Chicken erythrocyte. A low-magnification image of chicken erythrocytes (12 μm × 12 μm) clearly shows two individual cells (Fig. 3). The shape of the cells somewhat suggests the image of a derby hat (the inner part bulges upward, forming a convex area). The diameter of this convex surface is approximately 4 μm. Figure 4 shows a 500 nm × 500 nm area of a chicken erythrocyte, which also presents a series of blebs and holes on the surface. The measurements of these features are presented in Table 2. The holes in this sample are not as clear as those seen in the human erythrocyte.

TABLE 1. Dimensions of holes and blebs on the human erythrocyte surface

No.	
Length of longer axis of holes (nm)	
1	35
2	40
3	40
4	40
Length of longer and shorter axis of blebs (nm)	
1	110.70
2	80.45
3	55.45

Calotes erythrocyte. Figure 5, a low-magnification image (14 μ × 14 μ), shows a derby hat-shaped nucleated erythrocyte from a lizard. The bulging region spans a width of 7–8 μ, which is equivalent to the size of a human erythrocyte. Calotes are known to have larger erythrocytes compared to other species. Figure 6 at a higher magnification (500 nm × 500 nm) again reveals blebs and depressions. In this image, the areas of the holes and depressions are not amenable to easy measurement. Table 3 shows the size range of the blebs, which appear to be circular in contour.

Toad erythrocyte. The low-magnification image (14 μm × 14 μm) of the whole erythrocyte of a toad (Fig. 7) again shows a derby-shaped structure, with a bulge forming a convex surface spanning a region of about 5 μm in the middle of the erythrocyte. Figure 8 at higher magnification (500 nm × 500 nm) reveals blebs and holes on the surface. Table 4 provides data on the size class of these structures. In this specimen, we were able to measure both axes of the blebs.

Fish (Heteropneustes fossilis) erythrocyte. Figure 9 shows a low-magnification (10.24 μm × 10.24 μm) image of the fish erythrocyte, in which we can see that the central region is concave, spanning a length of 3 μm. The erythrocyte is distinctly elliptical (10 μm × 7 μm).

This normal shape (as defined by good optical microscopy images) again suggests that no excessive morpholog-

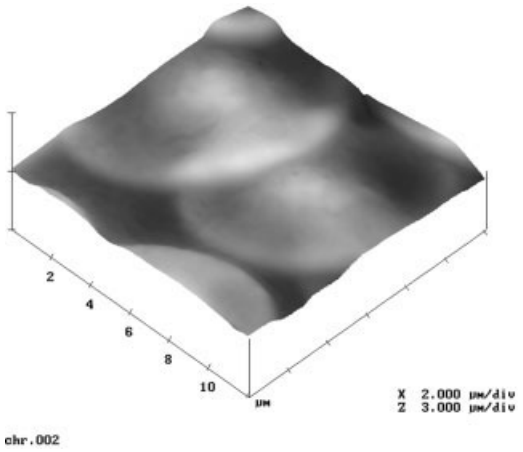

Fig. 3. AFM image of a chicken erythrocyte showing the normal shape (bulging out from the nuclear region).

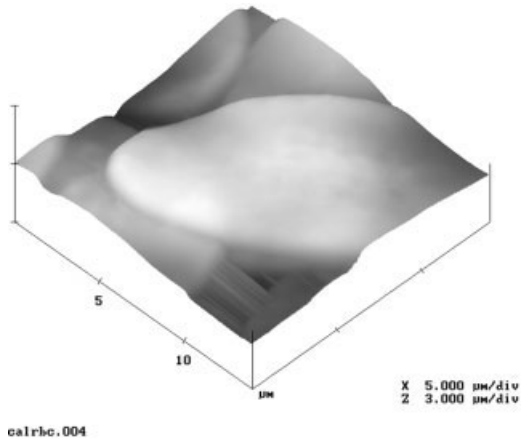

Fig. 5. AFM image of a calotes erythrocyte showing the bulge of the nuclear region.

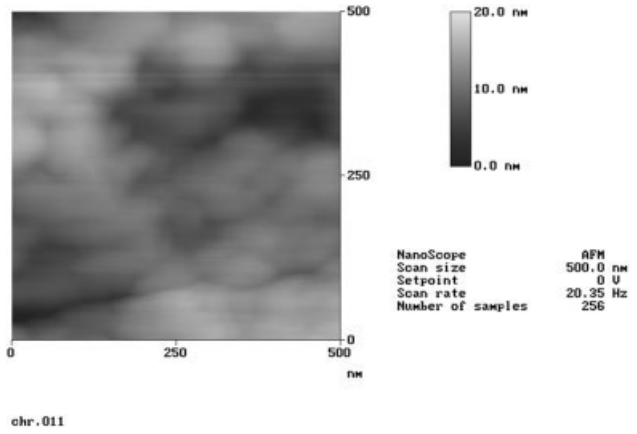

Fig. 4. AFM image of a chicken erythrocyte showing blebs and holes.

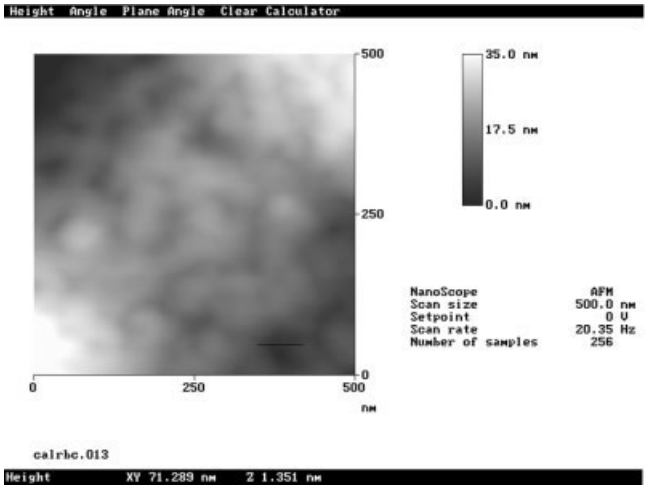

Fig. 6. AFM image of a calotes erythrocyte showing blebs and holes.

TABLE 2. Dimension of holes and blebs in a 500 nm × 500 nm area of chicken erythrocyte surface

No.	
Length of longer axis of holes (nm)	
1	432
2	342
Length of axis of blebs (nm)	
1	60
2	75
3	85

TABLE 3. Dimensions of blebs in a 500 nm × 500 nm area of calotes erythrocyte

No.	
1	75 nm
2	55 nm
3	50 nm

ical distortions occurred due to shear and stress during the drying procedure. Figure 10a depicts an image at higher magnification (540 nm × 540 nm), in which globular blebs are arranged in tiers around a hole. The approximate diameters of the holes and blebs are listed in Table 5. Here, too, some of the blebs are so clear that both the axes can be measured.

II LFM Images

Human. The appearance of human erythrocytes as revealed by LFM was previously described by Guha et al. (2002).

Toad. Figure 11a and b show the AFM image of a single erythrocyte (a) and the LFM image of the same cell (b). The image in Figure 11a is not as clear as that in Figure 7, but the derby hat shape is still recognizable. The LFM image (Fig. 11b) shows very clearly six different rough zones in the bulge (i.e., the area above the nucleus). The rest of the cell surface also reveals very clear zones, which appear as striations or bands.

Fish. Figure 12a and b likewise compare AFM and LFM images of the same erythrocyte. The nucleated region is depressed, but within this area regions of

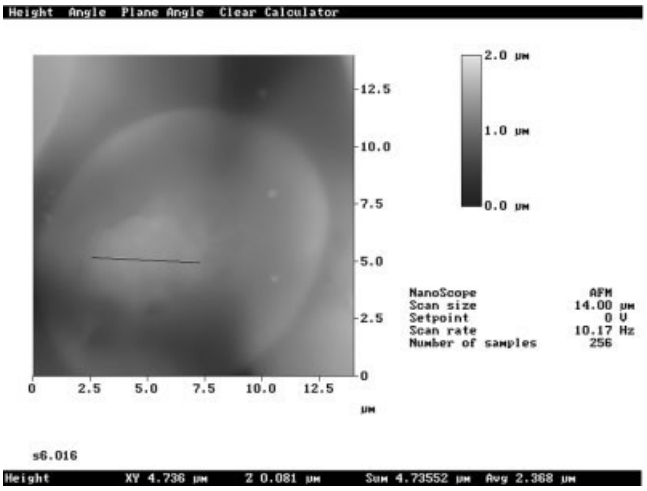

Fig. 7. AFM image of a toad erythrocyte showing bulging of the nuclear region.

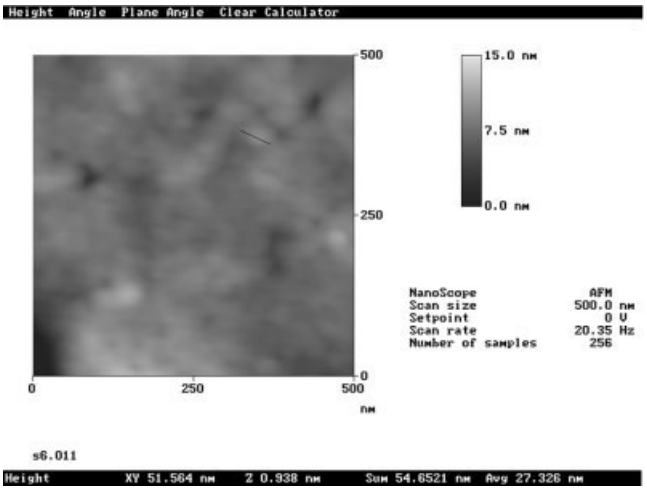

Fig. 8. AFM image of toad erythrocytes showing blebs and holes.

TABLE 4. Dimensions of holes and blebs in a 500 nm × 500 nm area of toad erythrocyte

No.		
Length of longer axis of holes (nm)		
1	40	
2	45	
3	50	
Length of two axes of blebs (nm)		
1	51	46
2	61	–
3	61	61
4	76	61
5	66	–

variable roughness are noticeable. This is also evident on the remaining part of the cell membrane. Similarly, Figure 10b shows the LFM image at a higher magnification.

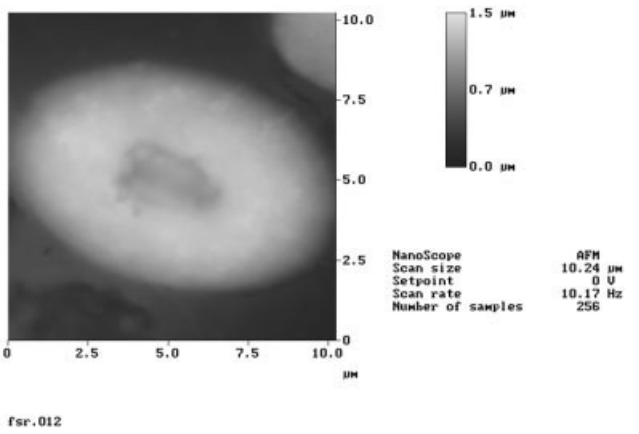

Fig. 9. AFM image of a fish erythrocyte. Here the nuclear region is clearly depressed.

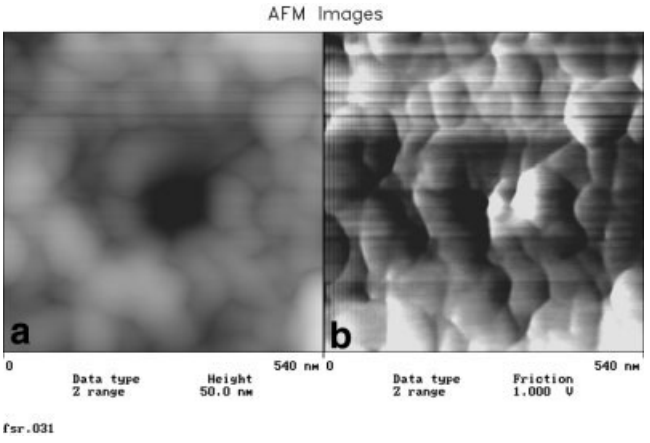

Fig. 10. **a**:AFM image of a fish erythrocyte showing blebs arranged around a hole in tiers. **b:** The corresponding LFM image (see below).

TABLE 5. Dimensions of holes and blebs in an area of 540 nm × 540 nm on the fish erythrocyte surface

No.		
Length of axis of holes (nm)		
1	108 nm	
2	<108 nm	
Length of two axes of blebs (nm)		
1	82	116
2	88	130
3	72	72

DISCUSSION

The investigations reported here were undertaken on the basis of two rationales: 1) the basic cytoarchitecture of erythrocytes has been conserved throughout the history of phylogenetic evolution, although it is possible that some variations in different species occurred; and 2) with the help of AFM we expect to obtain novel details at the ultrastructural level.

It is worthwhile to obtain AFM images of cells because at present this technique is known to be the best method

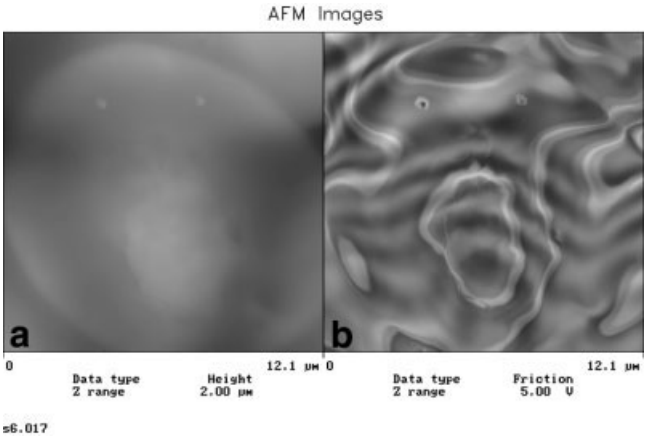

Fig. 11. AFM image of a toad erythrocyte (**a**), and an LFM image of the same cell (**b**), showing well-ordered strata of surface roughness.

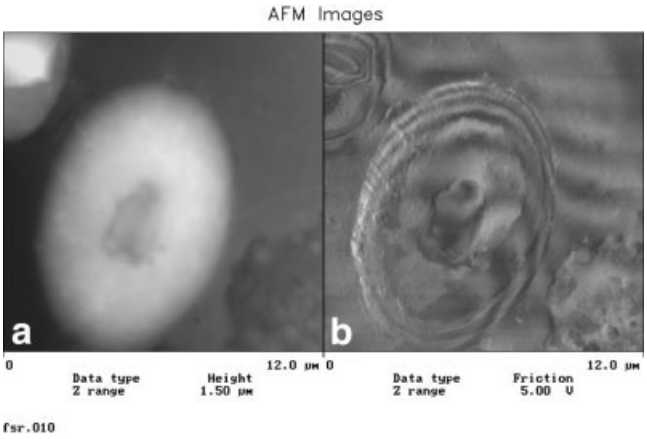

Fig. 12. AFM image of a fish erythrocyte (**a**), and an LFM image of the same cell (**b**).

for surface analysis. It requires no special treatment for preparation, and yields images at magnifications far exceeding those of SEM. The technique employed here (i.e., air-drying whole blood on a slide) is likely to involve forces of shear and stress, leading to morphological distortions; however, the images of whole erythrocytes (human or piscine) indicate very clearly that no great distortion occurred. With the exception of mammals (humans), all of the phyla examined are known to possess nucleated mature erythrocytes. The nuclear region in the chicken, reptile, and toad appears to be convex, while in the fish it apparently is concave.

More importantly, a conserved pattern of the erythrocyte surface (i.e., an arrangement of blebs and holes) apparently has persisted throughout phylogenetic evolution. This result supports the hypothesis that cytoarchitecture has been conserved. Zhang et al. (1995) described for the first time the highly uneven surface of the human erythrocyte as revealed by AFM. This surface can be described as a "hill and dale" pattern; however, in our image, at least some of the "dales" can be better described as "holes." In other words, the dales are not just lower than the hills—some of the dales are depressed below the baseline level. In the figures, a color-coded scale very roughly indicates the depth of some of these holes. An image of a lysed erythrocyte published by the Thermomicroscope Company (Sunnyvale, CA) suggests holes caused by the lattice of the spectrin-actin network that might roughly correspond to this system of depressions in the intact erythrocyte surface (Guha et al., 2002).

The results of this study emphasize that from fish to toad to reptile to chicken to human, a general surface pattern has been conserved. The dimensions of the holes and blebs are very variable, and the small sample size of this study precludes us from drawing certain conclusions (for example, as to whether holes in the fish erythrocyte are larger than those in the toad). However, we point out that the size range of all of the erythrocyte blebs is approximately 50–150 nm. This is admittedly a wide range, but it may be worth noting that none is as small as 4–10 nm, and none is as large as 300–500 nm. The blebs must represent the top surface of a cluster of protein/lipid molecules, which suggests that the cluster is within a certain size range. By analogy, one can consider the buckyballs of carbon atoms and the multimeric molecules of porphyrins and derivatives (Yokoyama et al., 2001). Future work may shed more light on these points.

An interesting question is whether the differences in erythrocyte membrane structure reflect the physiological/biochemical characteristics of the corresponding animals. It is known that humans habituated to high altitudes (e.g., in the Andean altiplano) have a much higher oxygen-binding capacity than individuals living on lower plains. AFM images of "within-species" samples that differ in physiological status, such as flightless birds and long-distance flying birds, might shed light on this aspect.

LFM is a powerful technique that reveals variable zones of roughness (as measured by varying frictional forces) on the erythrocyte surface. This was observed in a previous study of human erythrocytes (Guha et al., 2002), in which very clear banded zones of alternate high (whitish) and low (darker) roughness areas could be seen. In the present study, the normal AFM image shows a very uniform area on the surface of the fish erythrocyte (Fig. 10a), but the corresponding LFM reveals clear alternating regions of high and low roughness. This trend occurs even in the depressed (nuclear) region. An even more striking pattern is seen in the toad erythrocyte (Fig. 9b). It is satisfying to note the power of the LFM technique, with which we can explore new architectonic patterns on the erythrocyte surface.

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