Aspects on the relief of living surfaces using atomic force microscopy allow “art” to imitate nature

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Abstract

The visualization of the surface of biological samples using an atomic force microscope reveals features of the external relief and can resolve very fine and detailed features of the surface. We examined specimens from the skin of the amphibians Salamandra salamandra Linnaeus, 1758, Lyciasalamandra luschani basoglu Baran & Atatür, 1980 and Mesotriton alpestris Laurenti, 1768, and from the surface of pollen grains of the plant species Cyclamen graecum Link, 1835 and Cistus salviifolius Linnaeus, 1753, which exhibit certain interesting features, imaged at the nanoscale level. It is likely that the relief influences the attributes of the interfaces between the tissues and the environment. We found that the microsculpture increases in size the surface of the examined tissues and this might be particularly important for their performance in the field. Microsculpturing of amphibians’ skin may affect water regulation, dehydration and rehydration, and cutaneous gas exchange. Pollen grain relief might affect the firmness of the contact between pollen surface and water droplets. High resolution imaging of the external relief showed that roughening might induce wetting and influence the water status of the specimens. In addition, roughness affects the radius of water droplets retained in between the projections of the external relief. Roughness of the tissues was highly correlated with their vertical distance, whereas surface distances were highly correlated with horizontal distances. By enabling a more detailed characterization of the external sculptures, through sophisticated techniques, a more comprehensive examination of the samples indicates similarities among different living tissues, originated from different kingdoms, which can be attributed to environmental conditions and physiological circumstances.

Key words: amphibian, atomic force microscope, pollen, relief, surface.

INTRODUCTION

Recently, the study of biological surfaces introduced an active area of research (Bhushan 2009), which is related to the development of functional materials that are based on mimesis of several characteristics and properties. Structures with features’ dimensions ranging from the macroscale to the nanoscale are extremely common in nature (Bhushan 2009). Water regulation, self-cleaning, energy conversion and conservation, adhesion, coloration, thermal insulation, self-healing and sensory-aid mechanisms are some of the examples found in nature. Properties of biological surfaces result from a complex interplay
between surface morphology, miscroscope properties and environmental conditions. Research into the microsculpture of the functioning biological surfaces allows us to understand (through nanodevices and physiological processes) some inspired and designed adaptations of the species, under field conditions. Millions of years before man made manipulated synthetic structures, biological systems were using nanoscale architecture to produce striking features. Usually, biological surfaces are irregular. They possess 3-D complex structures by exhibiting a size spectrum ranging from microstructures far down into the nanometer range that can be observed using an atomic force microscope (AFM). Nowadays, several sophisticated techniques are available for investigation of the complex nature of living surfaces. AFM allows the surface of biological samples to be imaged at high resolution under physiological conditions (Ushiki et al. 1996; Round et al. 2000; Webster & Carpender 2002; Rhizopoulou et al. 2008).

“Art imitates nature” is a well known Aristotelian catchphrase (Aristotle, The Physics 194.a.21-22, Wicksteed & Cornford 1957), also been mentioned by his student Theophrastus (Theophrastus, The Causes of Plants II.18.2, Einarson & Link 1990) during the 4th century BC. According to Aristotle, the term “nature” is considered as the primary material out of which any natural object is made. Fundamental to Aristotle’s natural philosophy was his view about the goal-directed character of nature (Aristotle 4th century BC, Nicomachean Ethics 1100. b.30, Rackham 1934; Schummer 2001). Art and nature manifest certain general principles, concerning adaptation of form to function and a relationship between the end to be attained and the character of the matter necessary to attain it (Rorty 1980; Lloyd 1987). Aristotle contrasts the processes of art with those of nature by calling them “makings” (Aristotle 4th century BC, Metaphysics 1032.a.25-6, Tredennick & Armstrong 1935). An analogy can be detected between nature and art: because art (through human action) and nature are analogous, and because “making” is goal-directed, so too is nature (Aristotle 4th century BC, The Physics 199.a.1-20, Wicksteed & Cornford 1957).

Art imitates the ends of nature and such imitation should be a feature of the completion of nature; art, carrying nature forward to completion, would imitate by taking up nature’s end and pursuing its achievement (Granger 1993). Everything that is formed either by art or by nature exists in virtue of some proportion (Aristotle 4th century BC, Generation of Animals 767.a.17, Peck 1942).

The skin of amphibians is highly permeable to water and, as such, is important in their water economy. Furthermore, in all amphibians, the skin is connected to respiration, osmoregulation (Shoemaker & Nagy 1977), hydroregulation (Hillman 1980) and to a limited degree to thermoregulation, because the latter is complicated by the requirements of amphibians to maintain a moist skin (Brattstrom 1970; Brattstrom 1979). Some integumentary structures such as costal grooves or dermal flaps in some salamanders are associated with increased cutaneous vascularity (Lopez & Brodie 1977). Increased surface area plus vascularization function to increase water uptake in terrestrial amphibians, whereas increased surface area in aquatic amphibians provides for increased respiration (Spotila 1972; Duellman & Trueb 1994). The skin of amphibians, irrespective of taxonomic group or habitat, gives up water at approximately the same rate when exposed to equivalent conditions of desiccation. In contrast, rehydration rates are highly variable and depend on structural differences and absorptive properties of the skin, which seem to be related to habitat (Brown et al. 1977). Aristotle studied some traits of the external appearance of living organisms, which are related to their mode of living; he noticed that the differences between terrestrial, amphibian and aquatic animals are related to their respiratory organs (Aristotle 4th century BC, Historia Animalium 589.b.28, Peck 1965).

The surfaces of plants are multifunctional interfaces between the tissues and their environment (Karkkulova et al. 2008). The pollen of terrestrial species has well-developed exines with thick sculptured and basal layers (Cooper et al. 2000). At a microscopic level, however, pollen grains are quite distinct in size, shape and surface. The microsculpture of pollen structure forms is favorable for water absorption (Kesseler & Harley 2006). In addition, pollen grains are interesting from the material science point of view because the native polymer, the sporopollenin, found in the outer layer (exine), is one of the toughest known materials. Pollen grains are the male vector for reproduction of angiosperms. They are produced in great amounts by the stamens and are dispersed widely by wind, water, animals and insects. The strong envelope that contains the reproductive cell, that is, the exine, is characterized by the ornamentations of its surface, wall structure and the apertures. Almost every plant genus, and sometimes specific species, exhibits a particular exine that permits its identification. In considering the multitude of functions of this boundary layer, it is worth noting that it combines many aspects attributed to smart materials and the way it has evolved seems to be extraordinarily well suited to playing many different roles at a time. Subunits of pollen surface indicate various spaces among island-like structures that are interconnected to occupy the entire pollen exine (Xing et al. 2000).
In the present study we examine features of the microsculpture of the skin of three amphibian species and the outer surface of pollen grains from two plant species grown in the Mediterranean Basin. We attempt to identify traits on the surface sculpture that might influence their water behavior, in the context of the Mediterranean ecosystem. The present study applies an integrative research approach using biological samples, based on advanced scientific equipment, methodologically related with “art” inspired by Aristotle (Polymeni 2007, 2008; Rhizopoulou 2007, 2008).

MATERIAL AND METHODS

Experimental animals

Salamandra salamandra Linnaeus, 1758 is a terrestrial species widely distributed across Europe. It inhabits mainly deciduous and/or mixed forests, where it hides under logs, leaf litter and other places that provide shelter and moisture. It is mostly a nocturnal species, and the female releases the young larvae into water (Thorn 1968). In this work, samples of S. salamandra were taken from the island of Evvoia. Lyciasalamandra luschani basoglui Baran & Atatür, 1980 lives on the small, arid Greek island of Kastellorizo (Megisti) and on the neighboring coast of Turkey. Its habitat expands in Mediterranean maquis, phrygana and pine forests; areas with loose substrate and limestone crevices are preferred, providing humid places as shelter. It is mainly a nocturnal species, active during and after rainfall. L. l. basoglui is viviparous independent of water, giving birth to two fully developed young (Baran & Atatür 1980; Tzannetatou-Polymeni 1986; Polymeni 1994). Specimens of L. l. basoglui were collected on Kastellorizo Island, in the South-east Aegean Archipelago. Mesotriton alpestris Laurenti, 1768 is widely distributed in the central mainland of Greece, on the mountain range of Pindos and in Peloponnese, in both deep and shallow various water bodies located in a variety of mountainous habitats. M. alpestris is an oviparous species. Several cases of paedomorphosis are known. Variation of activity and, consequently, reproduction periods depend on latitude and altitude (Sotiropoulos et al. 1995; Valakos et al. 2008). M. alpestris specimens were collected from Mount Tymphi in Northern Greece.

Experimental plants

Cyclamen graecum Link, 1835 (Primulaceae) is a common autumn-flowering geophyte. The species is native to southern Greece, the Greek Islands, southern Turkey and Cyprus and blooms from September to November. Cistus salviifolius Linnaeus, 1753 (Cistaceae) is one of the most common of several types of Cistus species (rockroses), with beautiful white flowers that occur throughout the Mediterranean region; the species flowers between April and May in Greece. Mature pollen grains were collected from flowers of the two species that grew in an open field in Greece (38°57.5’ N, 23°48.0’ E). During the classical period, wreaths made of different types of rockroses were used to decorate altars of heroes (Rhizopoulou 2004).

Experimental procedure

A Tapping Mode AFM (TM-AFM, Veeco Instruments) was used for imaging of the amphibian’s skin and pollen surface over a 4 μm² to 1 μm² scan area of biological tissues. An AFM traces the surface topography of the sample and provides high resolution 3-D surface images of a sample. Among the advantages of AFM in examining biological tissues is its ability to provide information on surface properties. Quantitative information on the estimates of the microstructure of the examined surfaces is further obtainable from the AFM images. Although there are some innate limitations for AFM imaging, the AFM has great potential for providing valuable new information in histology. Various parameters were analyzed and processed, using the software package Nanoscope III (Veeco, USA), to detect detailed information on the microsculpture of the surface of the specimens. AFM provided detailed, quantitatively evaluated data for the horizontal and the vertical distance (nm) that represents the height of a step in the surface, between the folds (insert markers), the surface distance (nm), being the actual length between the two markers, and the roughness (nm) of the surface. The surface area ratio is the percentage of the 3-D surface area (2-D image) versus the projected flat surface area, onto the threshold plane. One more advantage of using AFM in this work is that the specimens could be used without any chemical treatment.

Data were subjected to analysis of variance that was carried out by using the statistical software package OriginPro 8 (OriginLab).

RESULTS AND DISCUSSION

Skin surfaces from two salamanders, S. salamandra (Fig. 1a) and L. l. basoglui (Fig. 1c), and one newt, M. alpestris (Fig. 1b), possess a similar pattern, as indicated by projections in the shape of peaks and cavities, which vary in height, density and arrangement (Table 1). In these tissues, the surface area ratio, which represents the density of the relief, was correlated with their roughness and the surface distance of the microsculpture was highly correlated with...
the horizontal distance (Table 1). In *S. salamandra*, a densely sculptured surface with a roughening of 37 nm, leads to an increased skin surface distance (135 nm) compared with the horizontal distance (109 nm) of the skin specimen. The skin of *L. l. basoglui* (Fig. 1c) exhibits the highest roughness (68 nm) among the three examined amphibian species, which in combination with deep cavities with vertical distances of 141 nm and exposed surfaces (where the horizontal distance is 367 nm and surface distance is 404 nm) favor the retaining of water for a prolonged period of time. The skin of *M. alpestris* (Fig. 1b) possesses a smoother surface (the roughness is 34 nm) than that of *L. l. basoglui*, although similar to that of *S. salamandra*. The density of forms of the abovementioned species projected per unit of surface (i.e. the surface ratio) possesses a quite similar arrangement on the surface of the skin, as indicated by the estimates of the surface ratio for *L. l. basoglui*, *S. salamandra* and *M. alpestris* (Table 1).

In considering the estimates of horizontal, vertical and surface distance of the skin relief (Table 1), it seems likely that the skin of *S. salamandra* (Fig. 1a) permits higher water loss than the skin of *L. l. basoglui* (Fig. 1c). *S. salamandra* is a terrestrial viviparous salamander that lives in forests, whereas *L. l. basoglui* is an endemic species of a small arid Greek island (Kastellorizon) in the South-East-

**Figure 1** Atomic force micrographs: 3-D profile of the surface (left), integrated line of measured points on plane profile (center) and profile view of the line section (right) with quantitative data (presented in the text). Skin microsculpture of *Salamandra salamandra* (a), *Mesotriton alpestris* (b) and *Lyciasalamandra luschani basoglui* (c). Pollen grain surface of *Cyclamen graecum* (d) and *Cistus salviifolius* (e).
ern Aegean Archipelago and the neighboring coast of Minor Asia. The species has become viviparous as a result of the deficit of water. These animals hide in underground burrows, cracks and crevices and dry-stone walls, in order to avoid dehydration (Tzannetatou-Polymeni 1986). It seems likely that their rough skin surface helps in retaining water during prolonged periods of hiding. Therefore, the epidermal sculpturing is important for water regulation (Lillywhite & Licht 1974; Lopez & Brodie 1977). Microsculpturing on the surface of amphibians’ skin (Fig. 1a–c) may affect oxygen consumption (cutaneous gas exchange), dehydration and rehydration, as well as to the creation of a film of air around the body, which could facilitate cutaneous respiration in the case of the newt *M. alpestris* (Fig. 1b).

Pollen is like a dust of plants, which bursts when moistened (Freer 2006). Pollen grains released from the anthers of mature flowers as individuals (Kesseler 2006) have evolved a multitude of complex mechanisms and structural components to actuate their expansion through the absorption of a drop of water (Messerli & Robinson 2003). Researchers have developed numerous sophisticated experimental methods to quantify the physical properties of plant tissues (Geitmann 2006). AFM enables very fine detailed study of the surfaces, while the microstructures and nanostructures of living surfaces have a great influence on their attributes as interfaces and exhibit interesting surface patterns (Bhushan 2009). The microsculpture of selected pollen grains from two plant species is shown in Fig. 1D and E. Such microsculptures may provide protection from damage by environmental factors (e.g. rain, wind and living organisms). The external surface of pollen grains of *C. graecum* (Fig. 1d) appears smooth (estimated roughness: 28 nm), whereas a tenfold higher roughness (263 nm) is detected on the surface of pollen grains of *C. salviifolius* (Fig. 1e); the roughness permits some wetting scenarios to be assumed (Herminghaus 2000; Wagner et al. 2003). Estimates of the surface of pollen of *C. graecum* differ substantially from those of *C. salviifolius* (Table 1). It is likely that different features of the surface at the nanoscale level are related to adaptations to different microclimatic conditions, mostly combined with the water availability in the field. The pollen grain relief of *C. graecum* (Fig. 1d) and *C. salviifolius* (Fig. 1e) may affect the firmness of the contact between pollen surface and water; pollen is attached to mating surfaces by wet adhesion, and, as a consequence, the surface of the grains expands (Heslop-Harrison 1970). The pollen microsculpture plays

| Table 1 Mean values of estimates of horizontal, vertical and surface distance, roughness and surface area ratio of the examined tissues, using atomic force microscopy |
|---------------------------------|-----------------|-----------------|-----------------|---------------|-----------------|
| Species                        | Horizontal distance (nm) | Vertical distance (nm) | Surface distance (nm) | Roughness (nm) | Surface area ratio (Sr) |
| *Salamandra salamandra*        | 109 ± 3           | 63 ± 1           | 135 ± 4           | 37 ± 2        | 1.3              |
| *Mesotriton alpestris*          | 312 ± 5           | 84 ± 3           | 335 ± 4           | 34 ± 3        | 1.3              |
| *Lyciasalamandra luschani basoglu* | 367 ± 2       | 141 ± 5          | 404 ± 6           | 68 ± 1        | 1.2              |
| *Cyclamen graecum*              | 172 ± 5           | 75 ± 2           | 200 ± 1           | 28 ± 1        | 1.8              |
| *Cistus salviifolius*           | 585 ± 1           | 397 ± 4          | 724 ± 8           | 263 ± 7       | 1.6              |

Source of variation: all species

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<td>Surface and vertical distances</td>
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<td>29.242</td>
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</table>

Source of variation: amphibians

| Surface area ratio and roughness | 0.007 | 156.481 | * |
| Surface and horizontal distances | 389.591 | 477.390 | ** |

Data presented here are means of five samples ± standard deviation. Analysis of variance between atomic force microscope (AFM) estimates of the micro-sculpturing of the different species; levels of significance (P) are shown as *P < 0.05, **P < 0.01 and ***P < 0.001.
CONCLUSIONS

Whereas the skin of the three amphibian species studied reveal approximately the same surface area ratios (Table 1), *L. I. basoglui* appears to have a higher value of roughness. Consequently, the radius of the water droplets retained in between the projections consisting the epidermal relief, is bigger than the radius of the droplets on the other two species. According to Kelvin’s equation concerning the vapor pressure of droplets, evaporation on the skin of *L. I. basoglui* takes place at a slower rate. In the case of pollen grains, quite similar values of surface roughness were observed on pollen of *C. graecum* (indicating the density of forms on the surface) are exhibited by *C. graecum*, with a low roughening, and *C. salviifolius*, with a high roughness. The detailed study via AFM, presented here, reveals that microsculptural patterns increase in size the surface volume of the tissues and this might be particularly important for their performance in the field. The observed relief on the surface of pollen grains is expected to affect the water absorption by the tissue. Smooth surfaces (increasing the water/surface contact area) were observed on pollen of *C. graecum*, which is an autumn flowering species, growing in the field under adequate water availability. Irregular surfaces (increasing the height of forms, water droplets penetrate into the depressions and wet the surface) were observed on pollen of *C. salviifolius*, which flowers in the Mediterranean ecosystem during spring at the onset of the prolonged drought period. Forms, detected via AFM, on the surface of tissues from the examined biological species reveal features linked to an astonishing functionality of the boundary layer. Hence, the tissues’ roughness was highly correlated (Table 1) with their vertical ratio (*r* = 0.99), horizontal (*r* = 0.88) and surface distance (*r* = 0.93); in addition, surface distance was linearly related with the horizontal (*r* = 0.99) and the vertical distance (*r* = 0.95). No significant correlations were found between surface area ratios and both vertical distances (*r* = 0.26) and roughening (*r* = 0.26) of the tissues. The relief, representing structural features at the microscale level, shows an aspect ratio of the horizontal versus the vertical distance, higher than one in the species mentioned above (i.e. 1.74 in *S. salamandra*, 3.72 in *M. aplestris*, 2.60 in *L. I. basoglui*, 2.29 in *C. graecum* and 1.47 in *C. salviifolius*).

The skin of *M. aplestris* and *L. I. basoglui* exhibited more extended surfaces in comparison with the narrower skin surfaces of *S. salamandra* that inhabits moist places. The same holds true for pollen of the spring flowering *C. salviifolius* that exhibited a smoother surface, when compared with the pollen surface of the autumnal flowering species *C. graecum*, which grows in crevices. It is probable that surface features, as indicated by submicron patterns, compared with water contact angle may be transferred to biomimetic materials (Bhushan 2009; Stratakis et al. 2009). Further investigation will be required to test this hypothesis.

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