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Atomic force microscopy study of living diatoms in ambient conditions

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Summary

We present the first *in vivo* study of diatoms using atomic force microscopy (AFM). Three chain-forming, benthic freshwater species – *Eunotia sudetica, Navicula seminulum* and a yet unidentified species – are directly imaged while growing on glass slides. Using the AFM, we imaged the topography of the diatom frustules at the nanometre range scale and we determined the thickness of the organic case enveloping the siliceous skeleton of the cell (10 nm). Imaging proved to be stable for several hours, thereby offering the possibility to study long-term dynamic changes, such as biomineralization or cell movement, as they occur. We also focused on the natural adhesives produced by these unicellular organisms to adhere to other cells or the substratum. Most man-made adhesives fail in wet conditions, owing to chemical modification of the adhesive or its substrate. Diatoms produce adhesives that are extremely strong and robust both in fresh- and in seawater environments. Our phase-imaging and force-pulling experiments reveal the characteristics of these natural adhesives that might be of use in designing man-made analogues that function in wet environments. Engineering stable underwater adhesives currently poses a major technical challenge.

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Introduction

Diatoms are unicellular microalgae with a cell wall consisting of a siliceous skeleton enveloped by an organic case essentially composed of polysaccharides and proteins (Hecky *et al.*, 1973). The cell walls form a pillbox-like shell (called the frustule) consisting of two valves that fit within each other with the help of a set of girdle bands. Frustules vary greatly in shape, ranging from box-shaped to cylindrical; they can be symmetrical as well as asymmetrical and exhibit an amazing diversity of nanostructured frameworks (Fig. 1).

Diatoms are found in both freshwater and marine environments, as well as in moist soils, and on moist surfaces. They are either freely floating (planktonic forms) or attached to a substrate (benthic forms), and some species may form chains of cells of varying length. Individual diatoms range from 2 μm up to several millimetres in size, although only few species are larger than 200 μm. Diatoms as a group are very diverse with 12 000–60 000 species reported (Werner, 1977; Gordon & Drum, 1994).

Currently, human chemical synthesis cannot produce siliceous structures with the hierarchical structural detail of the diatom frustules nor can ordered siliceous structures be produced synthetically under the benign conditions of diatom biomineralization. Biosilicification occurs at ambient temperatures and pressures, whereas artificial chemical synthesis of silica-based materials (e.g. resins, molecular sieves and catalysts) requires extreme conditions of temperature, pressure and pH.

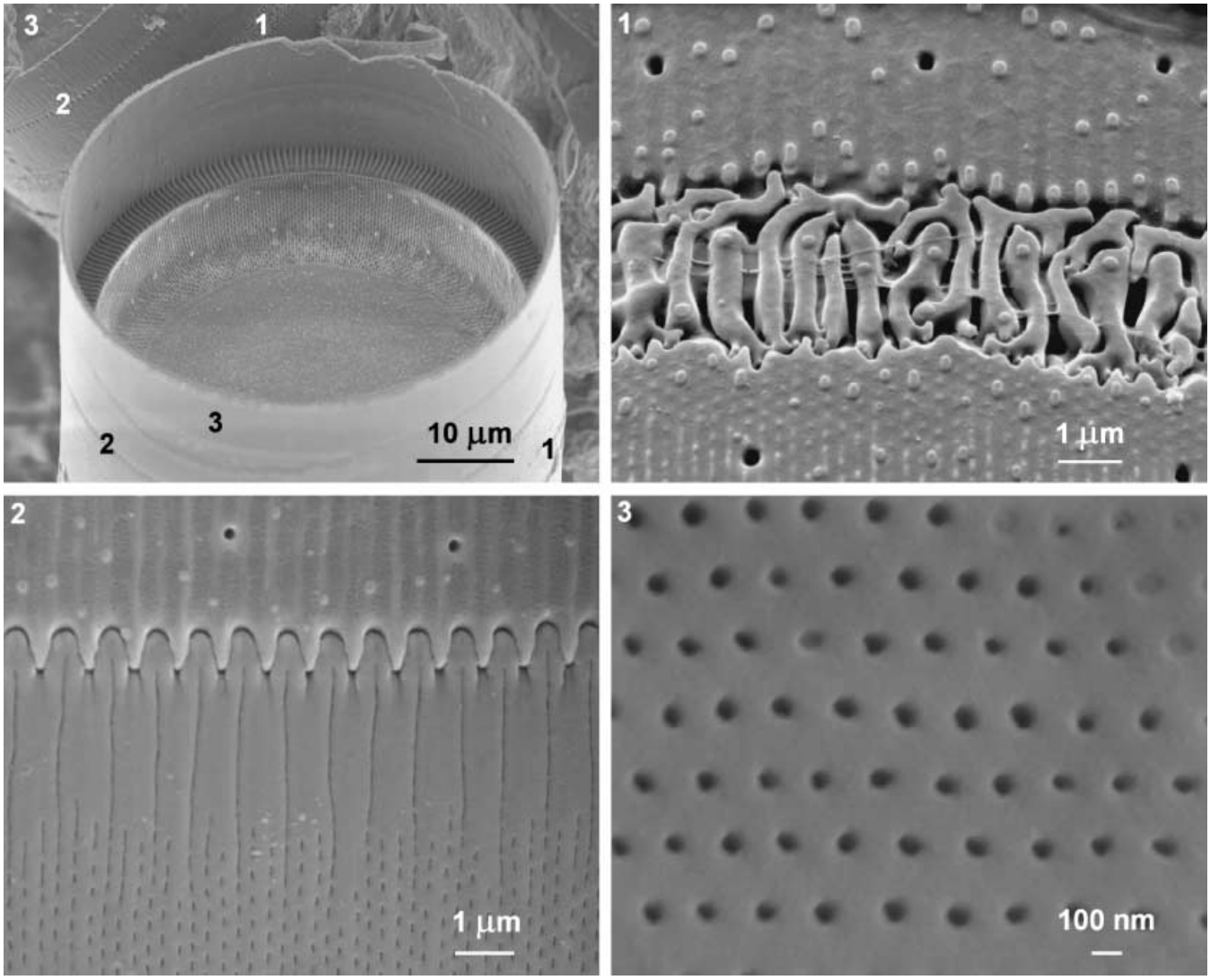

Fig. 1. Diatoms are unicellular organisms whose surface comprises nanostructured amorphous glass. Scanning electron micrographs of frustules of *Ellerbeckia arenaria*, a freshwater diatom, reveal amazingly beautiful and diverse details on a single cell (diatoms collected in Salzburg and kindly supplied by A. M. Schmid). The 'tower of life' (top left), the 'holy syllable OM written in glass' (top right), 'microman' complete with eyes, teeth and beard (bottom left) and nearly perfect hexagonal patterns (bottom right). The images on the top right and bottom are magnified regions denoted by the respective numbers in the top left image.

Linder *et al*. (1992) presented the first atomic force microscopy (AFM) study of diatoms. The surface structure of six different diatom species collected from a mud sample was imaged after the cells had been briefly rinsed with ethanol to kill, clean and immobilize them. In contrast to these still images, the present study demonstrates for the first time the ability to image living diatoms using the AFM. In contrast to conventional scanning electron microscopy (SEM), the samples do not have to be covered with an electrically conductive layer. Furthermore, the AFM allows for investigations of micromechanical properties of the cell surface, for example viscoeleastic properties, adhesion forces and hardness measurements. Further advantages of AFM are effortless sample preparation and unprecedented resolution on insulating materials.

The results from this AFM study provide important basic information to researchers working in biomimetics. Biomimetics is a new interdisciplinary research field seeking to understand the relationships between structures and functions of biological composites systems in order to design and synthesize new materials, possibly without the toxic residues characteristic of non-biological modes of industrial mass production (Mann, 1993; Sarikaya, 1994). Understanding the processes involved in biomineralization may eventually allow us to mimic these structures and produce optimized materials with minimal environmental impact.

Materials and methods

Imaging with the AFM requires a means of firmly attaching the sample to a 'holder' to permit stable scanning. Linder *et al.* (1992) and Almqvist *et al.* (2001) fixed dead diatoms to their respective sample holders. We used a different approach. By following a simple and effective strategy, we 'naturally selected' AFM-compatible diatom species from a number of species living in a freshwater aquarium. For that purpose, freshwater aquarium plants covered with benthic diatoms were placed in a jar filled with water, as well as two left-handed European freshwater snail species, *Physa fontinalis* and *Planorbarius corneus*, and some glass slides. In the following 3 weeks, the diatoms colonized the jar and the glass slides. The snails were feeding on the diatoms, predominantly leaving three species behind , which obviously strongly attached to the substrate: *Eunotia sudetica* (a diatom species that is abundant in acidic ponds but absent in alkaline environments; Beakes *et al.*, 1988), *Navicula seminulum* and a yet unidentified species. The glass slides were then transferred to an Si-enriched diatom growth solution (Diatom Medium, Culture Collection of Algae and Protozoa, Cumbria, U.K.; Beakes *et al.*, 1988) in order selectively to favour diatom growth, until they densely populated each slide. New samples can be prepared by adding clean glass slides and allowing the cells to colonize the new surface for about 2 weeks. Before imaging, the glass slides are rinsed with tap water to remove any green algae and debris.

Sample holders are vacuum mounted on a Zeiss Axiovert inverted microscope. Imaging is performed in tap water or diatom medium with a Digital Instruments BioScope with Nanoscope Controller Phase Extension Box (Digital Instruments, Santa Barbara, CA, U.S.A.) and a home-made tapping-mode fluid cell. Engaging the cantilever (Si_3N_4, spring constant $k = 0.06$ N m^{-1} and 0.12 N m^{-1}, Digital Instruments) takes place under optical control (the largest cells are about 50 µm in length and therefore easily visible in the inverted microscope).

Results and discussion

The AFM-compatible benthic freshwater diatom species selected by our 'natural selection' strategy are *Navicula seminulum* and *Eunotia sudetica* and a yet unidentified species. The natural adhesives of these cells, which attach them to the substrate as well as to each other (all of them are colonial forms), prove to be sufficiently strong that stable AFM imaging conditions are achieved without further sample preparation. The cells are imaged in their culture medium or in tap water while they are still growing on the glass slides. Tapping-mode as well as contact-mode imaging is easy to achieve as long as engaging the cantilever takes place on the cell surface. Engaging the cantilever on the glass slide might lead to problems with the *z*-range of the piezo, because the height of the cells can be several tens of micrometres, which might exceed maximum piezo extension.

Cell wall topography of living diatoms

Navicula seminulum grows in stacks of cells pointing out from the glass slide. These chains of cells can be about 10 cells high, as investigated by SEM (data not shown). Figure 2 reveals

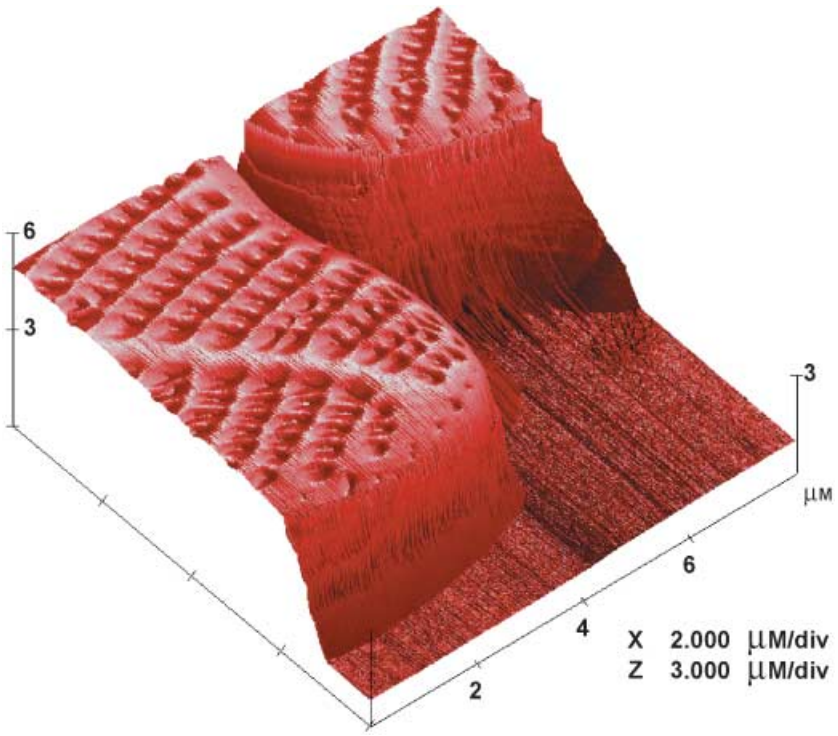

Fig. 2. Atomic force microscopy image of living *Navicula seminulum* cells. These benthic freshwater diatoms grow in tower-like colonies by adhesion of valve faces. The AFM image shows the top valve faces of adjacent cells of two different chains, growing on a glass slide. After imaging, the slides were put back into growth solution, and the diatoms continued to divide. Note that the flat area does not correspond to the surface of the glass slide, but is determined by the maximum possible extension of the *z*-piezo. Image acquired using AFM contact-mode imaging in water, imaging parameter topography, scan size 8×8 µm^2, scan frequency 1 Hz.

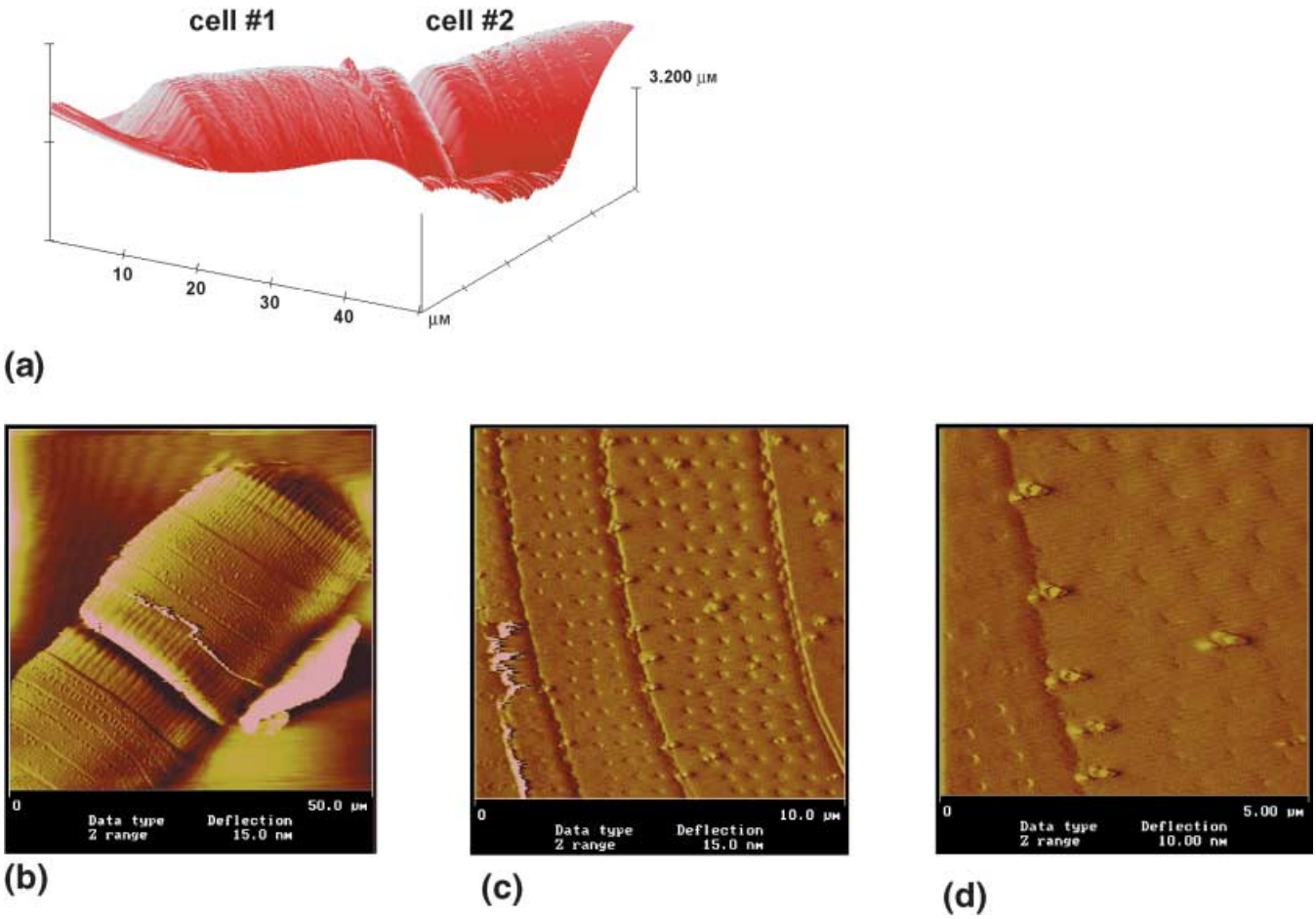

Fig. 3. (a) Topography three-dimensional representation and (b–d) deflection images of two cells of a stack of three of a yet unidentified diatom species. Contact mode in growth medium, topography and deflection, scan sizes $50 \times 50\ \mu m^2$, $10 \times 10\ \mu m^2$ and $5 \times 5\ \mu m^2$, scan frequency between 0.3 Hz and 1 Hz.

detailed surface patterning of the top valve faces of two adjacent cells of *Navicula seminulum*. Note that the flat area in the figure does not correspond to the surface of the glass slide, but is an artefact originating from the maximum piezo extension of our AFM.

The chains of *Eunotia sudetica* and of the yet unidentified species (see Fig. 3) grow with the valve faces perpendicular to the surface of the glass slide, allowing for AFM investigation of the girdle bands. Figure 3(a) shows the topography of two adjacent cells at the beginning of a chain of three cells (as seen in the light microscope) of the yet unidentified diatom species. The sides of both valves as well as the girdle bands from cell #1, the connecting region between the two cells and the side of one valve, and some of the girdle bands from cell #2 can be discriminated. The cells are alive and even continued to divide after imaging.

Girdle bands can telescope as cells elongate and grow. This might be visible in Fig. 3. The bead-like features on the edges of the girdle bands (Fig. 3b–d) are yet to be identified. This is the first time that such features have been seen. One possibility is that they are organic material that lubricates the connection between girdle bands. When a diatom cell divides it inherits both a valve and a set of girdle bands from the mother cell. Deposition of a new valve occurs prior to daughter cell separation, and then girdle bands are deposited sequentially beginning at the edge of the new valve. In Fig. 3, the new girdle bands would be those adjacent to the connection between daughter cells. We cannot tell whether any of these are new, but they all appear to overlap in the same direction. This suggests that they are all from the mother cell; this could be clarified if we knew how many girdle bands this species has. Note that the girdle bands on the opposite end of the cell are fully extended (i.e. they appear wider). The newest ones are more closely packed and are probably extending. This suggests to us that the beads are a lubricant because they only occur on the new bands. Identification of the diatom species will help to clarify this matter.

Determination of the thickness of the organic coating enveloping the siliceous skeleton

The siliceous skeleton of the diatom is enveloped by an organic coating consisting essentially of polysaccharides and proteins

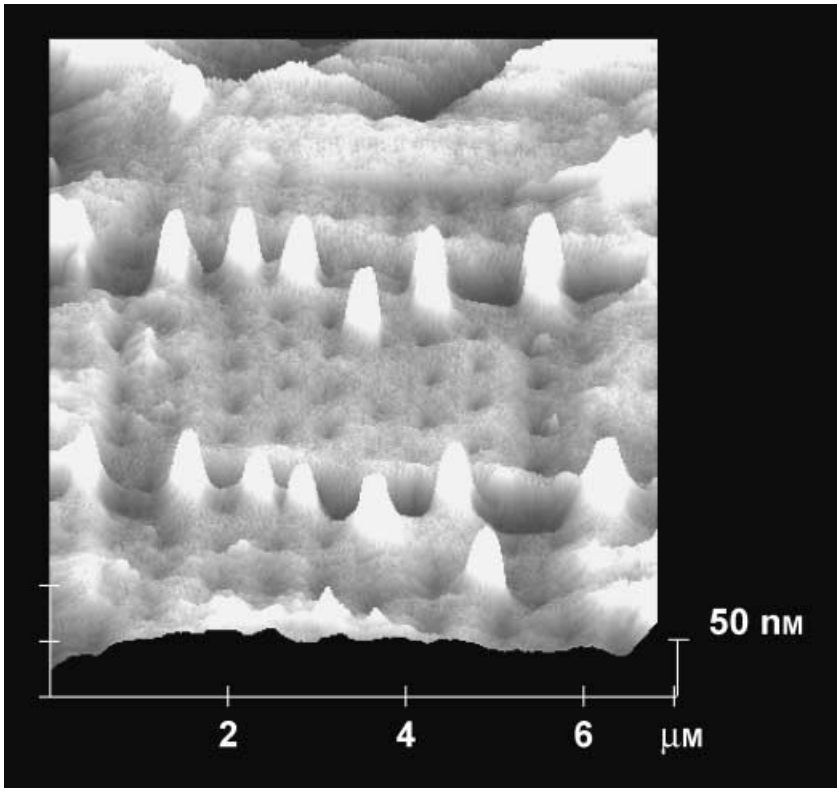

Fig. 4. Using the AFM cantilever as a tool. A 5×5-μm^2 area is repetitively scanned in AFM contact mode at high feedback force (about 100 nN). After 2 days, a slightly larger area comprising the area where the organic layer had died off meanwhile is imaged, revealing a height of the organic layer of about 10 nm. Contact mode, topography, scan size 7×7 μm^2, scan frequency 2 Hz.

and some lipids (Hecky *et al.*, 1973). A rough determination of the thickness of this layer is performed by following this protocol: a 5×5-μm^2 area is repetitively scanned with increased force set point (contact mode, about 100 nN) to mechanically remove and/or alter the organic material on this area, exposing the silica. After this, the cells are put back into their growth solution. Two days later, a larger area comprising the altered 5×5-μm^2 diatom surface is imaged with a force set point of about 1 nN. As can be seen in Fig. 4, the altered area, where the organic layer has been mechanically destroyed, is about 10 nm 'lower' than the unaltered surface. Experimental evidence reported by Volcani (1981) and TEM investigations by Leppard (1999) on extracellular polymeric substances corroborate this result.

Diatom adhesion and viscoelastic properties of the diatom frustule

Diatoms produce adhesives that are stable and robust in wet environments. All three diatom species investigated form chains by adhesion of their valve faces and are strongly attached to the glass slides, either with their valve face (*Navicula seminulum*) or with part of their girdle bands and valve sides (*Eunotia sudetica* and the yet unidentified diatom species).

Phase images depict the phase delay between the drive and response of the cantilever. These images contain information about the energy dissipated during the interaction of the AFM tip with the sample, and can help us to understand the viscoelastic and adhesion properties of the surfaces investigated, specifically of the organic material responsible for diatom adhesion For a review article on extracellular proteoglycans and extracellular polymeric substances see Lind *et al.* (1997).

Because phase imaging highlights edges and is not affected by large-scale height differences, it provides clearer observation of fine features that can be hidden by rough topography (compare Fig. 3a with Fig. 3b–d). To investigate the natural adhesives utilized to attach cells to each other and to the substratum, we tried to probe the cleft between two connected diatom cells with the AFM. In the yet unidentified species, the cleft at the cell–cell interface proved too deep. In this region, even the use of electron-beam-deposited AFM tips with high aspect ratio merely results in tip imaging (data not shown). Phase imaging reveals slight differences (2°) in viscoelastic and adhesion properties of the two adjacent valves.

Eunotia sudetica, by contrast, is very convenient for *in situ* investigation of the diatom adhesive at the cell interface, because there is barely any cleft between adjacent cells and valve undulations are less pronounced than in the other species investigated (Fig. 5a). The diatom adhesive is apparent as small topographic features at the cell interface. The bead-like structures are 10–20 nm high, have lateral dimensions of about 1 μm and are about 1 μm apart. The phase image clearly depicts the altered viscoelastic properties of these structures: the diatom adhesive causes a phase difference of up to 10° compared with the phase difference on the rest of each of the two frustules, where it is within 1° on each, apart from a 2° phase difference between the two adjacent valves (Fig. 5b), a

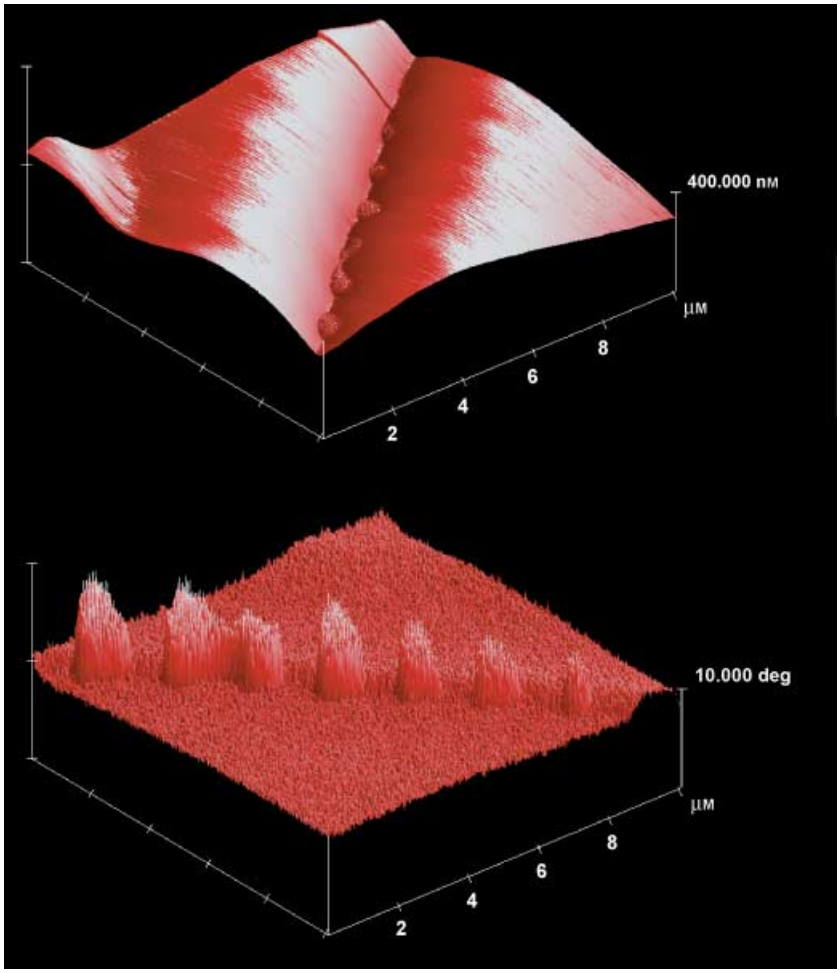

Fig. 5. (a) The adhesives in the contact region of two cells of *Eunotia sudetica* are apparent as small topographic features on the slightly undulated cell interface. The corrugation of the bead-like structures is between 10 and 20 nm, and their lateral dimension and spacing is about 1 µm. (b) In the phase image these features are far more striking. The diatom adhesive causes a phase lag of about 10° compared with the rest of the frustule surfaces, where on a single frustule it is within 1°. Note the 2° interfrustule phase step, which reveals slightly different viscoelastic properties of the two neighbouring valves. Tapping mode, topography and phase, scan size $10 \times 10\ \mu m^2$, scan rate 5 Hz. Note that for better view (b) is rotated clockwise by 90° as compared with (a).

feature which also appears in the other species, where the adhesives are not accessible because of deep clefts between the single organisms.

Force–distance curves on a natural and a man-made adhesive

Force–distance curves on double-sided sticky tape, on the surface and on the adhesive of *Eunotia sudetica* reveal basic differences in adhesion properties (see Fig. 6 for representative data). On double-sided sticky tape, the maximum adhesion force is about 15 nN; the molecules debond from the tip at a tip–surface separation of about 200 nm (Fig. 6a). The double-sided sticky adhesive quickly deteriorates in water, with the adhesion force decreasing within a few minutes (data not shown). On the diatom surface, no adhesion force can be detected (Fig. 6b). The diatom adhesive, by contrast, is strong and robust in the wet environment. To gain reproducible access to this natural adhesive, a chain of *Eunotia sudetica* that was embedded in a densely packed field of *Navicula seminulum* was scraped away from the glass slide with an STM-tip mounted on a three-dimensional micromanipulator. Over a period of several hours, force–distance curves were taken on the adhesive molecules that were used to attach the diatom cells to the glass slide. No change in the basic shape of the force–distance curves can be detected within hours of repetitive pulling in the area where the colony was located (see Fig. 6c,d for representative data). Typically, several debonding events occur until the natural adhesive molecules finally debond at a tip–surface separation of about 600 nm. For a detailed description of this study, see Gebeshuber *et al*. (2002).

Conclusions and outlook

Here, we present the first *in vivo* study of diatoms with AFM. Previous attempts to image living diatoms with AFM were not successful, because the cells did not attach strongly enough to the sample holders. Using natural selection involving freshwater snails feeding on algae we obtained – from a sample of numerous benthic freshwater diatom species growing on glass slides – three species that obviously produce outstanding natural adhesives: *Eunotia sudetica*, *Navicula seminulum* and a yet unidentified species. Such adhesives are strong enough to

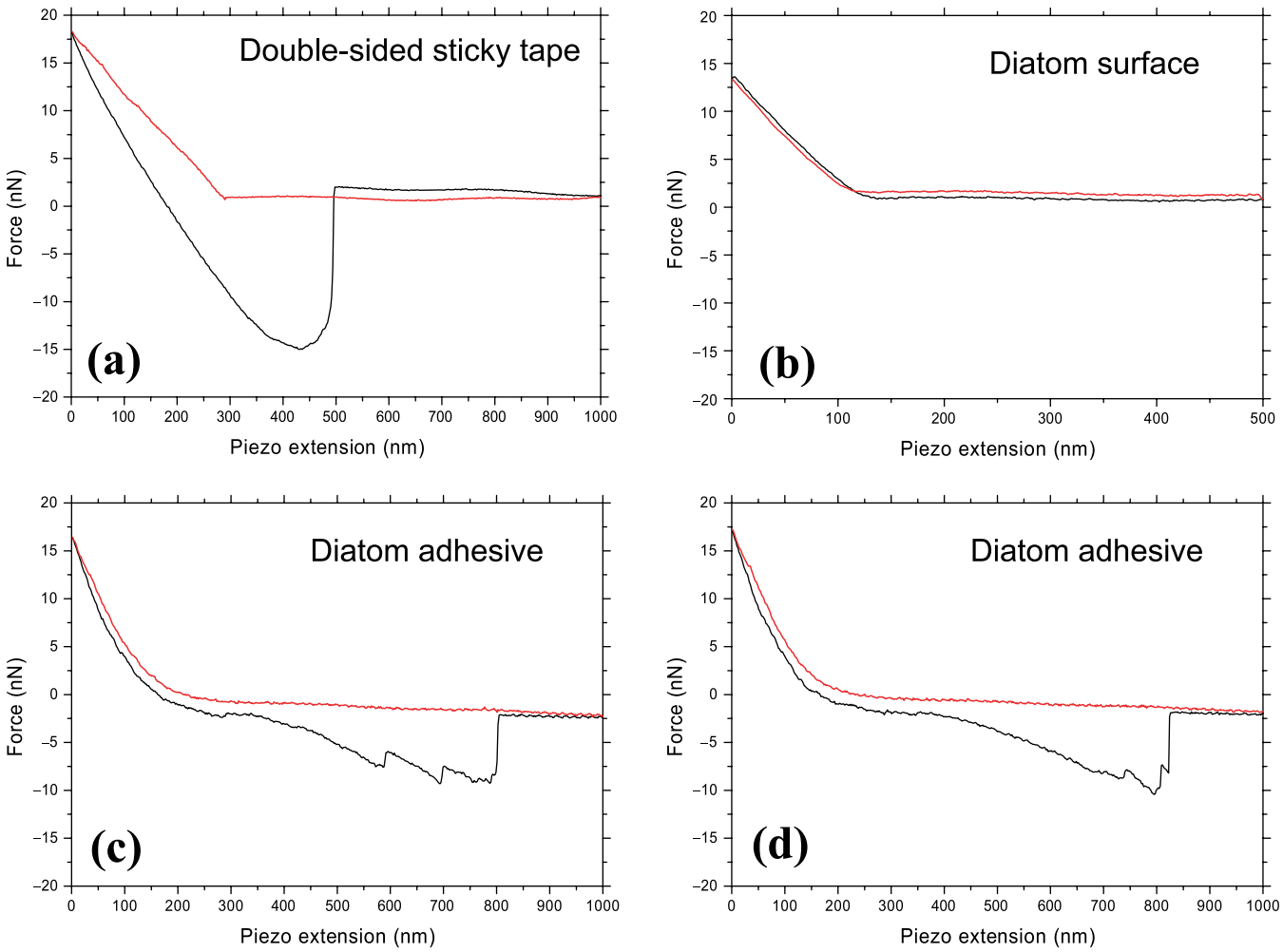

Fig. 6. Force–distance curves. (a) On double-sided sticky tape, the maximum adhesion force is 15 nN; debonding from the tip occurs at a tip–sample separation of about 200 nm. (b) No adhesion can be recognized on the diatom surface. (c,d) Representative data for the diatom adhesive that attaches *Eunotia sudetica* to the substrate. Note that several debonding events occur and that the pulling force must be applied over much larger extensions than in the double-sided sticky tape adhesive.

withstand the coarse snail grazing. The nanostructured siliceous shell of diatoms (e.g. Fig. 1) has been a favourite subject for assessing various aspects of the optical performance of microscopes (Dippel, 1882). In addition, diatom biogenic silica still poses a challenge for chemical synthesis or engineering. Our study includes the imaging of the cell wall of living cells (Figs 2–5) and using the cantilever as a tool to determine the thickness of the organic layer covering the siliceous skeleton (10 nm, Fig. 4). Furthermore, viscoelastic and adhesive properties of the frustule and diatom adhesion molecules as well as a man-made adhesive have been investigated (Figs 5 and 6). Phase imaging demonstrates rather uniform viscoelastic properties on the frustule, apart from the cell interface. Force pulling experiments reveal a strong and tough natural adhesive that is stable and robust in wet environments. Because imaging has proven to be stable over several hours, further *in vivo* AFM studies on diatoms will investigate dynamic biomineralization processes like the production of new frustules at the nanometre scale. These studies will provide important data regarding biomineralization. They might contribute to understanding the diatoms' solutions to challenges such as building – with environmental friendly conditions – nanostructured glass shells with high load capacity (Helmcke, 1985), engineering strong and robust adhesives that are stable in wet environments (Birchall, 1989), and the prevention of their silica shell dissolving in water owing to an organic layer. Modern technology is, for example, currently facing the problem that man-made glass-fibre-reinforced polymers show rapid quality deterioration when used in water (e.g. Connor *et al.*, 1997).

Furthermore, force pulling experiments will provide further data on the natural diatom adhesive at the molecular scale, perhaps resulting in new, more powerful adhesives, and also satisfy Sir Isaac Newton's comment in *Opticks* (1704):

'There are agents in Nature able to make the particles of joints stick together by very strong attraction, and it is the business of experimental philosophy to find them out.'

Acknowledgements

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