Identification of Nanopillars on the Cuticle of the Aquatic Larvae of the Drone Fly (Diptera: Syrphidae)

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Abstract

Here, we describe a nano-scale surface structure on the rat-tailed maggot, the aquatic larva of the Drone fly Eristalis tenax (L.). Larvae of this syrphid hover fly live in stagnant, anaerobic water-courses that are rich in organic matter. The larvae burrow into fetid slurry and feed on microorganisms which they filter out from the organic material. This environment is rich in bacteria, fungi and algae with the capacity to form biofilms that might develop on the larval surface and harm them. Using transmission and scanning electron microscopy we have identified an array of slender (typically <100 nm in diameter) nanopillars that cover the surface of the larvae. The high density and dimensions of these spine-like projections appear to make it difficult for bacteria to colonize the surface of the animal. This may interfere with the formation of biofilms and potentially act as a defence against bacterial infection.

Key words: Eristalis tenax, nanopillar, antibacterial, microtrichia

Nature has evolved a number of ways to reduce bio-fouling of surfaces. Amongst these, is superhydrophobicity, a state induced by the microscopic 3D structure of the animal or plant surface (Neithhuis and Barthlott 1997, Su et al. 2010). Such adaptations appear to be widespread, particularly on the air-surfaces of insect wings (Wagner et al. 1996, Fang 2007, Watson et al. 2010). Recently, such sculpted structural surfaces have also been shown to have inherent bactericidal properties. The surface structure of the wings of Psaltoda laripennis e.g., is covered by an hexagonal array of spherically-capped, conical 200-nm tall ‘nanopillar’ structures; spaced approximately 170 nm apart. Bacteria are killed when they come into contact with this material and this was shown to proceed by deformation of the outer surface of susceptible bacteria and their subsequent mechanical rupture (Hasan et al. 2012, Ivanova et al. 2012, Pogodin et al. 2013). Discovery of such surfaces has led to research into the development of biomimetic nano-materials with antiwetting and antibacterial properties (Guo et al. 2011, Hasan et al. 2013).

We have studied the larvae of the syrphid hover fly Eristalis tenax. The adult fly, which is found on every continent save Antarctica, is an important pollinator of yellow flowers, for which it has a preference, including agriculturally significant crops such as rape (Ilse 1949, Jauker and Wolters 2008). The larva mature in stagnant, fetid, anaerobic ponds, and water-courses where they filter-feed on the rich soup of bacteria associated with rotting organic material and faecal matter. They respire through long, telescoping, anal breathing tubes, or siphons, which reach from the floor of ponds to the surface, like a snorkel. We have examined larvae of the drone fly by transmission and scanning electron microscopy (TEM/SEM) and have identified an array of nano-scale spikes on the surface of the insect cuticle. We propose that these may have some function in preventing the formation of a bio-film on the animal which inhabits an incredibly high microbial pressure environment.

Materials and Methods

Collection of Specimens

Larvae were collected from a site in Surrey, south of London, UK over a 2-year period.

Transmission Electron Microscopy

Larvae collected directly from the wild were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.08M cacodylate buffer (Karnovsky’s fixative) at 4°C. They were not washed prior to fixation in order to preserve any biofilm associated with their bodies in their native state. After fixation they were washed three times in phosphate buffer and then osmicated by incubation with 1% osmium tetroxide in distilled H2O for 1h. Samples were then washed 3 × 10 min in ddH2O and dehydrated with a series of 30, 50, 70, 90, 3 × 100% alcohol and 2 × propylene oxide (at least 20 min each). They were infiltrated with 50% propylene oxide: 50% araldite resin overnight and with several changes of 100% resin the next day. Resin blocks were cured at 60°C overnight. Sections were prepared on a Leica Ultracut UCT microtome. Sections were sometimes...
counter-stained with Reynold’s lead citrate or with 0.5% aqueous uranyl acetate. Sections were viewed on a JEOL 1010 TEM (JEOLUSA MA, USA)

Protocol for Preservation of Delicate Structures for SEM
Samples were fixed, washed three times in phosphate buffer, osmicated, washed, and dehydrated into alcohol as in ‘Transmission Electron Microscopy’. The samples were then gently resuspended into hexamethyldisilazane (reagent grade >99%, Aldrich chemicals) and placed onto SEM stubs in a fume-hood. The volatile hexamethyldisilazane evaporated rapidly and preserved the delicate membrane-nanopillar structure of fragile specimens (Method developed by Dr P. Munro, personal communication).

Results
The Cuticle of E. tenax larvae is Covered in Spine-Like Nanopillars
We use a novel scanning electron microscopy preparation method in order to preserve the very thin, micron-scale features on cells (Dr P. Munro personal communication). We applied this technique to E. tenax larvae and were able to detect a novel form of nanopillar on the surface of the larval cuticle: e.g., a ~10 micron projection from the insect cuticle is shown in Figure 1A with these associated structures. Similar nanopillars were seen when other cuticle projections are sectioned for transmission electron microscopy (Fig. 1B). The nanopillars are of variable length and density (Fig. 1C and D). Some appear truncated or broken, but this may be mostly due to the spines being oriented at an angle to the plane of section.

Nanopillars are completely absent from the larval breathing siphon (Fig. 2A) The final section of this tri-partite structure is essentially an extension of two openings of the tracheal network aligned parallel to one another. It is covered in a very thin layer of almost featureless cuticle. At the junction between the two terminal parts of the siphon (Fig. 2A arrow and B) there were truncated nanopillars on the surface of the cuticle (dotted arrows). On some parts of the cuticle surface with these short nanopillar projections we identified bacterial biofilm (Fig. 2C). The larvae possess a number of claws on their fleshy prolegs (shown by SEM in Fig. 2D), these too are almost devoid of nanopillars (Fig. 2D and E).

Fine-Structure of the Nanopillars
Examination of ultrathin resin sections (thickness 50 nm) of the first larval instar of E. tenax by transmission electron microscopy revealed a thick, multilamellar cuticle covered by pagoda-like conical nanopillar projections (Fig. 3A). They are found at an approximate density of 16 per square micron of insect cuticle, though this varies between individuals and in different parts of the animal integument. This equates to an internanopillar distance of ~230 nm (SD = 98 nm). Nanopillars project approximately perpendicularly from the cuticle surface and vary in length from 200 to 1,000 nm, mean 728 ± 224 nm. Each is composed of a repeated unit, 19 ± 1.8-nm thick, composed of two elements 6.2 ± 0.9-nm thick, which, in some sections, appear to form the faces of a flattened disc, which have narrow, electron-lucent spaces in their central portion (Fig. 3B and C). Each disc is separated by a ‘spacer’ 3.2 ± 0.7-nm thick. Disc diameter steadily decreases with increasing distance from the larva (Fig. 3D), 60–90 nm at the base decreasing to <20 nm at the tips. After examination of many hundreds of nanopillars, we never identified any that became wider towards their tip at greater distance from the insect cuticle, confirming that this decrease in nanopillar diameter is not an artefact introduced by randomly sectioning through the tissue. The decrease in dimension follows a nonlinear pattern. Long nanopillars have a longer series of discs that only gradually diminish in width followed by more rapid decreases nearer the tip (Fig. 3D). There is little internal discernible structure to be seen when the nanopillars are viewed ‘en face’. The discs are not perfectly round; rather they have slightly undulate edges (Fig. 3E) which are marked by a discontinuous ring of increased electron density. This may reflect folds, superimposed in the projected plane of the section. This may explain why the discs appear to be discontinuously ‘connected’ along their entire edge (see arrows in Fig. 3C). There are no clearly identifiable microtubules or actin filaments in cross-section (as has been described for elaborate insect mechanosensilla (Wolfram 1990, Keil 1997) and they are uninnervated, having no discernible connection to cells beneath the underlying cuticle.

Nanopillars May Prevent the Adhesion of Bacteria
The number of adherent bacteria (classified as those within 30 nm of cuticle surface) and the ‘density’ of nanopillars along sections of cuticle were estimated by transmission electron microscopy. We use the number of nanopillars per unit cuticle length observed in sections as a proxy for the number of nanopillars per unit area on the two dimensional surface of the cuticle. A TEM image is a projection of a 50 nm-thick section and depending upon its orientation and how the section has cut through it, only part of the nanopillar may appear in this projection. In broadly ‘en face’ sections (e.g., that shown in Fig. 1D), it is apparent that the nanopillars are arranged in an array in which each individual spine is surrounded, approximately equidistantly, by 5, 6, or more others. Given the complexity of the cuticle surface topology, the variation in the arrangement and number of nanopillars and the limitations of TEM sectioning, we were unable to more precisely define the packing arrangement of the pillars on the cuticle surface. The number of bacteria adherent to the cuticle surface tended to increase in regions in which the nanopillars were more sparsely arranged (Fig. 4A), reaching a plateau. In regions of high nanopillar ‘density’ (more than four nanopillars observed every 1,000 nm of cuticle length) there were almost no adherent bacteria (arrow in Fig. 4A and B). Larger organisms that may contribute to developing biofilms appear to have been prevented from forming an attachment to the cuticle surface even when the density of nanopillars is quite low (e.g., the fungal hypha shown in Fig. 4C). At this lower density of nanopillars, however, smaller bacteria were able to adhere to the surface in the gaps between the nanopillars (Fig. 4D). Encapsulated bacteria appeared to be entangled between nanopillars (Fig. 4E and F). Here, nanopillars were shown to be deformable, as at the point of contact with the bacterial surface they appeared bent and in some cases folded-over.

Discussion
The surface cuticles of insects are elaborated by a number of complex excrescences. Setae, e.g., are micrometer-scale, high aspect-ratio (long-thin) hairs which project from the cuticle surface from shallow, pit-like structures. Microtrichia are much shorter projections, usually in the scale of 1–3 µm which sometimes form a dense ‘carpet’ on the surface of the cuticle. Setae are often associated with sensory functions whilst microtrichia are thought to be involved in either maintaining the water repellent properties of the cuticle (Goodwyn et al. 2008) or with the formation of a layer of trapped
air (plastron) in the case of aquatic insects (Cassie and Baxter 1944, Gao and Jiang 2004). In some cases the combination of both setae and underlying microtrichia contribute to this process. Microtrichia are thought to be derived from bundles of actin filaments originating from underlying epithelial cells. The processes we have identified do not conform to classical microtrichia; being substantially shorter and clearly composed of stacked arrays of disc-like material. We see no continuity with underlying cells and they do not appear to have a filamentous core. They are of variable length, are at least partly flexible and appear to break off readily. For this reason, we refer to them as nanopillars to distinguish them from classic microtrichia.

The density of nanopillars on the surface is almost as high as has been measured for the superhydrophobic surfaces of certain insect wings (Wagner et al. 1996, Hasan et al. 2012, Ivanova et al. 2012). However, the drone fly larva is aquatic and appears to be fully wettable (though we have not explicitly studied this). *E. tenax* nanopillars are high aspect-ratio projections and as such are unlikely to act
Fig. 2. Some areas of the insect cuticle surface are devoid of nanopillars. (A) A low power scanning electron micrograph of the breathing snorkel of a larva (arrow indicates the region seen in close up in B). (B) Nanopillars on the cuticle of the body of the larva (arrows) but there are none on the breathing siphon. (C) A spiny projection of a larger larva covered in sessile bacteria (white arrows). Exposed areas of the cuticle without bacteria have visible nanopillars (arrow heads). (D) The proleg of the larva: individual claws on the pro-leg appear sharp and featureless. (E) Transmission electron micrograph of a section through the claw reveals that they are mostly devoid of nanopillars (arrow); though these are clearly seen on the surrounding cuticle (larger arrow heads).
The nanopillars show novel ultra-structural features. (A) A projection on the surface of a first instar larva covered in nanopillars. Periodic banding is clearly visible. (B and C) In some sections the banding can be resolved into a series of smaller discs (blue) separated by a spacer which appear to be connected at their rims at some points (black arrows) but not at others (white arrow). At the very base of the nanopillar the discs are unfused and are a different size to the rest of the nanopillar (red). (D) A dot plot showing the distribution of the widths of 20 nanopillars measured from their tip (narrow point) to the base (attachment point on the cuticle). They are of variable length and show a non-linear pattern of change in diameter. (E) A transverse section through a nanopillar disc reveals granular structure with regions of increased electron density around the periphery of the disc (arrows).
Fig. 4. There is a correlation between nanopillar density and development of biofilm on the cuticle surface. (A) Dot plot showing the number of attached bacteria per micrometer cuticle length plotted against the average distance between the nanopillars. Each point represents data from a single transmission electron micrograph taken at random from parts of three larval insect cuticles. (B) Very high density of nanopillars in a cleft on the larval surface. (C) An alga is prevented from finding purchase on the surface of the cuticle by an array of nanopillars. (D) A region of low nanopillar density has allowed a number of bacteria to colonize the cuticle surface. (E and F) Bacteria caught up on the nanopillars. Note the deformation of some of the nanopillars indicating they have a certain inherent flexibility.
as waterproofing surfaces, unlike the conical, low aspect-ratio nanopillars identified on superhydrophobic cuticles. A high aspect-ratio may promote the wettability of the insect surface due to increased adhesion between water and the internanopillar cuticle. The insect spends a certain amount of time suspended from the surface meniscus but does not appear to pick up a layer of air; instead it swims down into organic material and breaths through its extended siphon. The nanopillars, being <6 µm long and held at right-angles to the cuticle are too short and inappropriately orientated to promote formation of a bona fide plastron (Goodwyn et al. 2008).

Although the nanopillars do not appear to promote superhydrophobicity; they do, however, appear to antagonize formation of a biofilm on the larval surface. Insects are thought to lack adaptive immunity, instead relying on innate strategies involving, in part, physical barriers to infection (Hoffman et al. 1996). Areas of the insect cuticle on which the nanopillars are highly abundant are completely free of adherent bacteria, fungi, and algae. Only when the center-to-center internanopillar gap increases above 250 nm do bacteria and other microorganisms find purchase. Although the larvae spend much of their time buried in foetid material at the bottom of water courses, they do, periodically, come to the surface. At such times they perform a complex series of writhing movements. Given the density of nanopillars on their surface we speculate that such movements could comb off or physically crush or pierce the bacteria trapped between the folds of the cuticle. The entire surface of the organism is not protected by such projections. They are almost entirely absent from the siphon and claws of the prolegs. This may be a result of abrasion but could also be the result of some specialization of the cuticle in this region (it is very thick e.g., on the claws). The main function of the nanopillars is likely to be to prevent colonization of the insect by offering a physical barrier between microorganisms and the cuticle, in much the same way that antipigeon spikes prevent pigeons nesting in niches on buildings. Even if bacteria could fit between these spines and attach to the surface, the presence of neighboring spines may interfere with biofilm formation as there is insufficient room for the bacteria to successfully divide or form productive interactions with other colonizing microbes. Given the growing interest in development of biomimetic antibacterial surfaces (Guo et al. 2011, Hasan et al. 2013), these results may be useful, offering an alternative geometry for production of nanoscale antibacterial surfaces. A number of interesting questions remain: we do not know if these nanopillars have any inherent bactericidal properties and, given their atypical structure, we do not know how they are formed.

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