Brochosomal coats turn leafhopper (Insecta, Hemiptera, Cicadellidae) integument to superhydrophobic state

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Leafhoppers (Insecta, Hemiptera, Cicadellidae) actively coat their integuments with brochosomes, hollow proteinaceous spheres of usually 200–700 nm in diameter, with honeycombed walls. The coats have been previously suggested to act as a water-repellent and anti-adhesive protective barrier against the insect’s own exudates. We estimated their wettability through contact angle (CA) measurements of water, diiodomethane, ethylene glycol and ethanol on detached wings of the leafhoppers Alnetoidia alneti, Athysanus argentarius and Cicadella viridis. Intact brochosome-coated integuments were repellent to all test liquids, except ethanol, and exhibited superhydrophobicity, with the average water CAs of 165–172°, and the apparent surface free energy (SFE) estimates not exceeding 0.74 mN m⁻¹. By contrast, the integuments from which brochosomes were removed with a peeling technique using fluid polyvinylsiloxane displayed water CAs of only 103–129° and SFEs above 20 mN m⁻¹. Observations of water-sprayed wings in a cryo-scanning electron microscope confirmed that brochosomal coats prevented water from contacting the integument. Their superhydrophobic properties appear to result from fractal roughness, which dramatically reduces the area of contact with high-surface-tension liquids, including, presumably, leafhopper exudates.

1. Introduction

Surface textures, formed by integuments or their lipid secretions, waxes, have been previously reported to be responsible for reduction of wettability in insects and plants [1–6]. Here, we report a similar function for micro-sized proteinaceous powder actively applied onto the body by representatives of the insect family Cicadellidae.

Members of the highly diverse family Cicadellidae, or leafhoppers, are unique in having their integuments coated with intricately structured particles known as brochosomes [7–10], for review see Rakitov [11]. In most leafhopper species, brochosomes are spherical honeycombs, 200–700 nm in diameter, with pentagonal and hexagonal wall compartments open into the hollow core (figure 1a–e). Brochosomes are composed of proteins, possibly with additional components, and are produced in vast numbers inside specialized cells of the Malpighian tubules (insect primary excretory organs, associated with the digestive tract, in some insects additionally performing synthetic functions), where they undergo gradual maturation within Golgi-derived vesicles [12–15]. Soon after moulting, leafhopper adults and, in some lineages, also immatures release colloidal suspension of brochosomes through the hindgut and apply it with their legs onto the new integument. Once the liquid has dried out, brochosomes are further spread during bouts of grooming behaviour, in which leafhoppers rapidly brush their integuments with the legs bearing comb-like rows of specialized setae; as a result, the entire integument becomes coated with a thin pruinose layer of brochosomes [11,16–21].

Several authors noticed that the integuments of leafhoppers are water-repellent and argued that brochosomal coats can protect these predominantly small and delicate sap-sucking insects from getting trapped into water and their own liquid exudates [11,16–18,21]. However, the wettability of leafhopper
integuments has not yet been subject to a quantitative study, and the alleged role of brochosomes has not yet been experimentally demonstrated. The goal of this study was, therefore, to experimentally characterize the wettability properties of leafhopper integuments coated and not coated with brochosomes.

2. Material and methods

(a) Insects
Adults of three leafhopper species representing different subfamilies, diets and habitats were collected in Kiel and Kronshagen, the state of Schleswig-Holstein, Northern Germany: (i) *Alnetoidia alneti* (Dahlbom; the subfamily Typhlocybinae, feed on mesophyll cell contents); occurs on tree leaves; forewing length 3.1–3.5 mm. Specimens were collected from *Acer campestris* L.; (ii) *Athysanus argentarius* Metcalf (the subfamily Deltocephalinae, feed on phloem sap); occurs in dry and mesic grasslands; forewing length 5.5–6.7 mm; (iii) *Cicadella viridis* (L.) (the subfamily Cicadellinae, feed on xylem sap) occurs on wetlands and mesic grasslands; forewing length 5.2–6.6 mm.

Prior to experiments, leafhoppers were kept in the laboratory in 30 × 30 × 30 cm meshed cages (LiveMonarch, USA) on cuttings of host plants standing in water.

(b) Wing preparations
Because measuring static contact angles (CAs) of liquid droplets requires that the studied surface is flat, we focused our experiments on wings. Wings of leafhoppers anaesthetized with carbon dioxide were carefully detached at the bases and glued flat onto ca 6 × 10 mm rectangular chunks of glass microscope slides with a thin layer of commercial Rimmel 60-s nail polish (Coty Inc., UK). For convenience, such individual wing preparations were arranged into linear series on microscope slides coated with double-sided sticky tape (see electronic supplementary material, figure S1).

![Figure 1. Structure of brochosomes. (a,b) Model of a typical brochosome, based on present results and published ultrastructural data [12,13], a three-dimensional reconstruction by O. Karengina, showing a general view (a) and cross-section (b). (c) Brochosome on the surface of eye of *Athysanus argentarius*, note that the wall compartment in the centre allows seeing through the entire hollow particle. (d) Brochosomes of *Alnetoidia alneti* accidentally trapped between two layers of polyvinylsiloxane and torn in halves when one layer was peeled off; note the holes connecting the central core with the outside. (e) Brochosomes of *A. argentarius*; the touching particles are in fact connected. (f) Brochosomal coat on the intact hindwing of *A. alneti*. (g) Same, brochosomes interconnected in branching chains. Scale bars: 50 nm (a,b), 100 nm (c–e) and 1 μm (f,g).](http://rspb.royalsocietypublishing.org/)

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Resting leafhoppers keep their wings folded over the abdomen. The dorsal forewing side is exposed and, therefore, is most likely to have protective properties but at the same time is most prone to surface damage, whereas the ventral forewing side and the hindwings are concealed, except during flight. To account for potential variation in wettability between different body parts, in each of the studied species, we examined three surfaces: the dorsal and ventral sides of the forewing, referred to below as the dorsal and ventral forewing, respectively, and the dorsal side of the hindwing, referred to as the hindwing.

(c) Removal of brochosomes

To remove brochosomes from the integument, we developed a peeling-off technique using polyvinylsiloxane (PVS) elastomer. This silicone-based material has been used for preparing impressions of micro-sculptured surfaces [22,23]. We used a Coltene President light body kit, art. no. 4667, from Coltene/Whaledent Inc. (USA), which includes two components, the base and the catalyst, combined in equal volumes to create a mixture polymerizing within 4–5 min. The wings glued onto the glass and the catalyst, referred to below as the dorsal and ventral forewing, respectively, and the dorsal side of the hindwing, referred to as the hindwing.

(d) Freshly moulted (naturally bare) specimens

Adult leafhoppers apply brochosomes onto their bodies before they start feeding, typically within the first 3 h after the moult [19]. In the interim, the new integument is naturally free of brochosomes. Therefore, to prove that our technique for removal of brochosomes using PVS did not affect other properties of the integument, we examined wings of four freshly moulted A. alneti adults. On these specimens, only CAs of water were measured.

(e) Measuring contact angles and estimation of apparent surface free energy

Surface wettability is commonly characterized by measuring CAs with standard well-characterized liquids and calculating from these measurements the surface free energy (SFE), an integral characteristic that takes into account both chemistry and geometry of the surface [24]. To emphasize that the CAs of liquid droplets are observed on the rough instead of ideally flat surface, these are referred to as the apparent CAs and the corresponding energies as the apparent SFEs [25].

To examine wettability of brochosomal coats and bare integuments, we measured apparent static CAs of water, ethylene glycol, diiodomethane and ethanol. On artificially bared and naturally bare wings of A. alneti, measuring CAs of ethylene glycol and diiodomethane were not practical because, in this small species, keeping the droplets from spreading beyond the wing area proved not possible. Ethanol droplets spread outside the wing area on all tested surfaces; thus estimating their CAs proved also impossible.

We used an OCAH 200 high-speed CA-measuring system (DataPhysics Instruments GmbH, Filderstadt, Germany) fitted with silanized glass capillaries (diameter of the tip = 0.15 mm). Static CAs were measured using the ‘needle in’ (in the case of unwettable surfaces; see electronic supplementary material, figure S2) or ‘sessile drop’ (wettable surfaces) methods with circle- or ellipse-fitting. The drop volume was 1 µl. The contact between the liquid and insect surface was documented by taking still images or video. A clean glass plate was used as a reference surface. The air temperature during the experiments was 24.8 ± 2.0°C, and the relative humidity was 50.4 ± 7.1% (mean ± s.d.).

For each individual leafhopper, CAs were measured on one dorsal forewing, one ventral forewing and one hindwing. On each wing, only one droplet was measured, and the same liquid, after removal of brochosomes. The number of examined wings for each combination of wing type, liquid and treatment (intact or bared) varied from 5 to 15 (mean, 9.4); for exact numbers, see electronic supplementary material, tables S1–S3.

The apparent SFE and its components were calculated from the CAs of water, ethylene glycol and diiodomethane using the universal Owens–Wendt–Kaelble method [26] as implemented in SCA20 software (DataPhysics Instruments). For additional theory and comparison of alternative methods, see Zenkiewicz [24].

(f) Scanning electron microscopy

For ultrastructural observations, we used a Hitachi S-4800 cryo-scanning electron microscope (cryo-SEM; Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan Inc., Abingdon, UK). To examine the structure of brochosomes, brochosomal coats and bare wings, glass chunks with wings prepared for CA measurements were glued onto SEM metal stubs with a double-sided carbon tape, sputter-coated with gold–palladium (3–6 nm), and examined in the microscope’s standard mode at accelerating voltages of 3–5 kV.

Previous cryo-SEM studies demonstrated that interactions of hydrophilic and hydrophobic surfaces with water droplets can be visualized at high resolution upon freezing of the sample [27]. To obtain close-up views of brochosomal coats interacting with water droplets, intact leafhopper wings were glued onto a metal holder using Tissue-Tek OCT Compound (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands), the holder was attached to the SEM’s insertion rod, finely sprayed with distilled water from a 30 cm distance from a mist bottle, immediately dipped into liquid nitrogen (~196°C), and inserted into the SEM’s plasm centre. The sample was transferred into the cryo-preparation chamber, sputter-coated with 6 nm of gold–palladium, and examined in the SEM in the frozen state at 5 kV and ~120°C. Alternatively, the holder with insect specimens was cooled down on dry ice, then taken off the ice (which prompted condensation of atmospheric water vapour on the surface), immediately dipped into liquid nitrogen, and then processed as above.

3. Results

3.1 Morphology of brochosomes and brochosomal coats

The brochosomes of all three species were highly similar in both structure and size. The diameter of the particles was 0.43 ± 0.044 µm for A. alneti, 0.50 ± 0.084 µm for A. argentarius and 0.49 ± 0.061 µm for C. viridis (mean ± s.d. of mean); the brochosomes of A. alneti were significantly smaller than those of either of the other two species (p < 0.00001), while the difference between the latter was not significant (p = 0.30). Most particles appeared hollow, with the air-filled core visible through perforated bottoms of the wall compartments (figure 1c,e). The internal structure was visible on several particles inadvertently torn open during removal of PVS layer (figure 1d).

The brochosomal coats were similarly uniform and dense in all the wings studied (figures 1f and 2a–c). Brochosomes
formed no visible bonds to the surface, but were often interconnected in chains of variable length, sometimes branching or forming loops (figure 1g).

(b) Morphology of bare integuments
Surface peeling with PVS resulted in a complete or nearly complete removal of brochosomes, with no more than a few isolated particles remaining, while at the same time preserving fine details of the integument proper, including setae and microsculpture (figure 2d–l).

The dorsal forewings of all three species displayed shallow shagreen texture at the submicron scale (figure 2d,g,j). In A. alneti, the only additional surface features were well-spaced conical microtrichia (both height and basal diameter between 0.5 and 0.8 μm, figure 2d; electronic supplementary material, figure S3a) and occasional rosette-like coeloconic sensilla. By contrast, in both C. viridis and A. argentarius, the dorsal forewings lacked microtrichia but bore larger structures. In C. viridis, they were covered with sparse scattered setae, 10–30 μm in length, and numerous (ca 530 per mm²) pit-shaped coeloconic sensilla of ca 3 μm in diameter (see electronic supplementary material, figure S3b). On the dorsal forewings of A. argentarius, similar setae were present along the veins.

The ventral forewings and the hindwings of all three species lacked any submicron textures, bearing only conical microtrichia and smoothed wrinkles (figure 2e,h,i,k,l). In C. viridis, the basal one-third of the hindwing was additionally sculptured with needle- and plate-like wax crystals (not illustrated); this area was excluded from the wettability assay.

(c) Wettability of brochosomal coats and bared integuments
The static CAs of water, diiodomethane and ethylene glycol on the assayed integuments are summarized in figure 3 and electronic supplementary material, tables S1–S3. For each integument type and each liquid, the values on the intact surfaces were significantly larger (p < 0.00001) than on the bared surfaces. Compared with these differences, the differences between body parts and between species, for a given surface type, were small, although some of these were statistically significant (p < 0.05; electronic supplementary material, table S4). On the intact wings, the
average CAs of water droplets varied between 164.9–167.8° for *A. alneti*, 170.0–172.3° for *A. argentarius* (see electronic supplementary material, figure S2), and 166.6–169.5° for *C. viridis*. The analogous measurements on the bared wings were 112.8–116.2° for *A. alneti*, 107.6–128.9° for *A. argentarius* and 103.1–114.9° for *C. viridis* (see electronic supplementary material, table S1).

The CAs of ethylene glycol and diiodomethane on brochosomal coats were 152.7–164.1° and 148.2–156.0°, respectively (figure 3a; see also electronic supplementary material, table S2). On the bared wings, the CAs were much lower, and the difference between the two liquids was more distinct (81.4–90.3° for ethylene glycol, 64.2–73.9° for diiodomethane; figure 3b).

In contrast to the other three liquids, on both intact and bared wings, ethanol droplets quickly spread across and beyond the wing area, which was interpreted as full wetting.

Estimates of the apparent SFE for the examined surfaces ranged between 0.09 and 0.74 mN m$^{-2}$ for the intact brochosomal coats (figure 4a) and between 20.0 and 34.1 mN m$^{-2}$ for the bared wings (figure 4b). In both cases, the dispersion surface energy was the dominant component.

During measuring of CAs, we noticed small amounts of brochosomes apparently getting stuck to the surface of water droplets. The specimen stage allowed us to bring the droplet in contact with the same integument area multiple times by moving the specimen up and down. Such repeated contacts resulted in the surface becoming increasingly wettable, apparently due to a progressive wear of the brochosomal coat (see electronic supplementary material, movie S1).

### (d) Wettability of naturally bare integuments

The static CAs of water droplets on the naturally bare hindwings (*n* = 4) and dorsal forewings (*n* = 3) of freshly moulted *A. alneti*, 119.9 ± 7.56° and 124.9 ± 7.97°, respectively, were larger than those on the artificially bared wings of that species: 113.53 ± 7.97° and 112.8 ± 8.60°, respectively (mean ± s.d.; electronic supplementary material, table S1). The differences proved to be significant in a two-way ANOVA with ‘condition’ (intact, bared or naturally bare) and ‘wing type’ (dorsal forewing, hindwing) as the parameters (*p* < 0.0001).

### (e) Interactions between microscopic water droplets and brochosomal coats, visualized in cryo-SEM

We observed multiple frozen droplets of water, between 20 and 200 μm in size, attached to the integuments (figure 5a–c). Most sites of attachment were cracks, sutures, veins and other features that created discontinuity of the brochosomal coat. The observed CAs of droplets varied from superhydrophobic (figure 5a) to hydrophobic (figure 5c). Almost invariably, the surface of the frozen droplet carried pieces of the brochosomal coat removed from the adjacent integument (figure 5b–d).

### 4. Discussion

We have demonstrated that brochosomes form superhydrophobic coats of leafhopper integuments with water CAs above 164° and apparent SFEs not exceeding 0.74 mN m$^{-2}$. The structure and properties of the coats were similar in adults of three species representing different subfamilies of Cicadellidae and different types of diet. Thus, our results can be considered to be representative of integumental brochosomal coats in general.
Figure 4. Estimates of apparent surface free energy for intact (a) and bared (b) leafhopper integuments, partitioned into polar (white) and dispersion (black) components. FWD, forewings, dorsal surface; FWV, forewings, ventral surface; HW, hindwings, dorsal surface. Note the scale difference between (a,b).

Figure 5. Leafhopper integuments sprayed with water, frozen in liquid nitrogen, sputter-coated with Au–Pa, and visualized in a cryo-scanning electron microscope. (a) Frozen droplet reveals the superhydrophobic state of the hindwing of Athysanus argentarius; the metal coat of the droplet has multiple cracks; the light powder on the lower half of the droplet is brochosomes; the droplet is anchored to a wing vein. (b) Frozen droplet situated on top of the claval suture on the forewing of Alnetoidia alneti; note the darker area of the integument, from which brochosomes were collected onto the surface of the droplet. (c) Frozen droplet anchored to the claval suture next to a broken wing area; the metal coat of the droplet has multiple cracks; the light powder on the lower front side of the droplet is brochosomes collected from the integument. (d) Same, close-up; note chains of brochosomes. Scale bars: (a–c) 50 μm, (d) 2 μm.
The observed similarity between CAs of water on naturally bare (freshly moulded) and PVS-bared wings of *A. aheti* generally validates our use of PVS for demonstrating that the superhydrophobicity of leafhopper integuments is created by brochosomes. The slightly lower water-repellence of artificially bared integuments probably reflects chemical differences between freshly moulded and older cuticles.

The estimated extremely low SFE suggests that brochosomal coats are repellent to high-surface-tension liquids, such as water and aqueous solutions. Similarly, low SFEs below 1.0 mN m\(^{-1}\) have been previously estimated for some artificial superhydrophobic surfaces [25]. The dominant dispersive component suggests that the surface of brochosomes contains a low proportion of polar molecules and that, given the same surface tension, brochosomal coats are more repellent to polar liquids. In our experiments, they were most strongly repellent to water (surface tension = 72.1 mN m\(^{-1}\), dispersion component = 19.9 mN m\(^{-1}\), polar component = 52.2 mN m\(^{-1}\) [28]), less so to ethylene glycol (surf. tension = 48.0 mN m\(^{-1}\), disp. comp. = 29.0 mN m\(^{-1}\), pol. comp. = 19.0 mN m\(^{-1}\) [29]), and least yet to diiodomethane (surf. tension = 50.0 mN m\(^{-1}\), disp. comp. = 47.4 mN m\(^{-1}\), pol. comp. = 2.6 mN m\(^{-1}\) [28]) (figure 3a).

The structure of brochosomal coats suggests the same mechanism of water-repellence as was previously found in many biological surfaces [3–5, 30–34]: rough surface texture allows the solid and the liquid to contact only at the tips of asperities while being separated elsewhere by a film of trapped air, which is known as the ‘Cassie state’ [35–37]. Superhydrophobicity and self-cleaning effect are typically attained on the surfaces that combine roughness at both the micro- and nanoscales [4–6, 38–40]; this principle of hierarchical roughness has increasingly been applied to the design of artificial surfaces [34, 37–44]. Similarly, in brochosomal coats, the microscale roughness formed by whole particles and their ensembles is combined with the nanoscale roughness created by the reticulate surface of individual particles. In fact, the structure of brochosomal coats is strikingly similar to that of artificial superhydrophobic coats prepared from raspberry-like silica-based submicron spheres [45, 46], the main difference being that brochosomes have the concave and raspberry-like particles, the convex sculpture. While technology is often inspired by biology [34, 47], in this particular case, the rapidly evolving technology appears to have inadvertently reinvented an organic solution.

The structure of brochosomal coats also resembles artificial surfaces with re-entrant sculpture, unwettable by both high-surface-tension liquids and low-surface-tension liquids, such as alcohols and alkanes, and therefore referred to as omniphobic [48, 49]. However, we observed that brochosomal coats were completely wettable with ethanol (surf. tension = 22.3 mN m\(^{-1}\)) [50]). Wettability with low-surface-tension liquids has also been observed on water-repellent plant surfaces [6].

Among related insects of the order Hemiptera, fine textures formed by close-set submicron processes on the wings of some cicada species (Cicadoidea) produce water CAs of up to 146° [51] (for an unrelated potential function of these processes, see Ivanova *et al.* [52]). By contrast, bare leafhopper wings exhibit poorly developed microsculpture (figure 2d–I) and moderately hydrophobic water CAs of 103–129°. CAs in that range have been recorded on other poorly sculptured insect integuments [1–3]. Nevertheless, leafhoppers attain water CAs well above the superhydrophobic threshold of 150° by coating their integuments with brochosomes. Such water CAs are among the largest observed among animals [3, 32, 33, 53] and plants [5, 31]. What sets the superhydrophobic coat of brochosomes apart from such previously studied biological surfaces is that it is applied onto the body actively, on demand, during specialized behaviour [11, 17, 19, 20] and is composed predominantly or exclusively of protein [11, 14, 15].

It appears that such strong water-repellence in leafhoppers—a group of exclusively terrestrial insects—primarily serves as protection from contamination by or getting trapped into liquid exudates produced by leafhoppers as a consequence of their feeding on plant sap [11, 16–18, 21]. The physico-chemical properties of leafhopper exudates remain unstudied. However, those of xylem-feeding leafhoppers (such as *C. viridis*) are close in composition to pure water [54, 55], and those of phloem-feeding species (such as *A. argentinarius*) contain excess sugars and are expected to be similar to honeydew of other phloem-sucking hemipterans, such as aphids. Using the capillary method, Pike *et al.* [56] estimated the surface tension of honeydew produced by the aphid *Pemphigus spirithoae* as 50 mN m\(^{-1}\). In this study, two liquids with the surface tension of 48 and 50 mN m\(^{-1}\); non-polar diiodomethane and polar ethylene glycol, respectively, exhibited superhydrophobic CAs (greater than 150°) on brochosomal coats, suggesting that the latter are repellent to honeydew as well.

Previous cryo-SEM studies demonstrated that frozen water droplets retain a nearly spherical shape on strongly hydrophobic surfaces [27]. Therefore, our cryo-SEM observations (figure 5a–d) confirmed the superhydrophobicity of brochosomal coats. We assume that most droplets that did not get anchored to local discontinuities of brochosomal coats, such as wing cracks (figure 5c), had rolled off the wings prior to examination. Additionally, these observations demonstrated the loose nature of brochosomal coats, indicated by the fact that the droplets collected brochosomes off the integument onto their surface (figure 5c–d). Such erosion of the coat explains the decrease of the observed CA when the water droplet was repeatedly brought in contact with the same area of the wing (see electronic supplementary material, movie S1). Because brochosomal coats are easily erodible, they can additionally reduce the risk of the leafhopper being captured by arthropod predators relying in their capture mechanisms on adhesion (adhesive pads of spiders and some predatory insects, sticky orb webs of spiders). Upon contact with adhesive surfaces, brochosomal coats will be easily detached from the leafhopper integument and will contaminate and disable the adhesive surface. Similar easily erodible structures are known from some carnivorous plants of the genus *Nepenthes*, where they are responsible for slippery properties of the insect traps [57].

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