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Direct evidence of phospholipids in gecko footprints and spatula–substrate contact interface detected using surface-sensitive spectroscopy

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Observers ranging from Aristotle to young children have long marvelled at the ability of geckos to cling to walls and ceilings. Detailed studies have revealed that geckos are 'sticky' without the use of glue or suction devices. Instead, a gecko's stickiness derives from van der Waals interactions between proteinaceous hairs called setae and substrate. Here, we present surprising evidence that although geckos do not use glue, a residue is transferred on surfaces as they walk—geckos leave *footprints*. Using matrix-free nano-assisted laser desorption-ionization mass spectrometry, we identified the residue as phospholipids with phosphocholine head groups. Moreover, interface-sensitive sum-frequency generation spectroscopy revealed predominantly hydrophobic methyl and methylene groups and the complete absence of water at the contact interface between a gecko toe pad and the substrate. The presence of lipids has never been considered in current models of gecko adhesion. Our analysis of gecko footprints and the toe pad–substrate interface has significant consequences for models of gecko adhesion and by extension, the design of synthetic mimics.

Keywords: geckos; adhesion; spectroscopy; NALDI imaging; sum-frequency generation spectroscopy; lipids

1. INTRODUCTION

Geckos have a remarkable ability to climb vertical walls and their adhesive and self-cleaning capabilities have fascinated biologists and materials scientists interested in developing synthetic mimics [1–8]. Geckos' feet have microscopic hairs called setae, which further split into hundreds of smaller structures called spatulae. When pressed into contact with any surface, the spatulae deform, enabling molecular contact over large areas, thus translating weak van der Waals interactions into enormous attractive forces. The setae and spatula are composed of β-keratin [9,10], which provides the toughness and resilience to support forces that are orders of magnitude higher than a gecko's body weight. Previous studies on gecko adhesion have only considered β-keratin hairs making contact with the surface, where the peeling process preserves the keratinized structure. Although not visible to the naked eye, we find that the geckos also leave behind footprints on glass they have walked across. Because degradation of the keratinized adhesive structures has never been observed, the presence of a footprint raises many intriguing questions. Firstly, what is the material that is wearing away during adhesive contact and being left behind in the form of a gecko footprint? Secondly, what are the implications for gecko adhesion as we know it?

Here, we used a combination of surface-sensitive spectroscopic techniques to identify the chemical nature of the residue left behind on solid substrates and assess its presence at the contact interface. Using nano-assisted laser desorption mass spectrometry (NALDI-MS), we have identified the residue as phospholipids with phosphocholine head groups. The chemical mapping of the footprint indicates that these phospholipids are left behind where the microscopic hairs make contact with the surface. Interface-sensitive sum-frequency generation spectroscopy (SFG) revealed predominantly hydrophobic methyl and methylene groups and the complete absence of water at the contact interface between a gecko toe pad and the substrate. These observations shed light on several outstanding questions regarding the influence of water on gecko adhesion [11–13].

2. EXPERIMENTAL METHODS

2.1. Gecko experiments

Before imaging, NALDI and SFG experiments, an adult (4 to 5 years old) Tokay gecko's (*Gekko*

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gecko) feet were cleaned by taking advantage of the innate self-cleaning properties of the setae. The animal was allowed to climb approximately 50 steps on a cleaned glass panel or manipulated to adhere to a clean glass panel repeatedly 50 times, with the exception of the gecko used in the freshly shed experiments. The glass panel was cleaned by first rinsing in a base bath, followed by a deionized water wash and then dried in an oven overnight.

2.2. Moisture condensation imaging

A gecko, approximately one week post moult, was induced to walk horizontally along a glass panel. After the gecko walked on the clean glass, the glass panel was placed in a room at 98 per cent humidity, allowing moisture to condense on the glass surface. The glass panel became opaque as a result of tiny water droplets covering the surface. The outline of footprints was revealed by differential condensation around the footprints. The image was then captured by a digital camera. A similar image was obtained by freezing a glass slide with a footprint and allowing the frozen water layer to melt and form liquid around the footprints.

2.3. Nano-assisted laser desorption-ionization mass spectroscopy

NALDI-MS and imaging experiments were performed with a Bruker UltraFlex III tandem time-of-flight (ToF/ToF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). This instrument uses a Nd : YAG laser in conjunction with a third harmonic crystal emitting at a wavelength of 355 nm and a frequency of 100 Hz. All spectra were measured in positive reflectron mode. The instrument was calibrated externally with a poly(ethylene glycol) standard prior to each measurement. Gecko toes with fresh and three-week-old setae were gently pressed on the NALDI target plate for footprint imaging. Gloves were worn at all times to prevent any contamination from the fingerprints of the experimenters. In addition, we analysed the fingerprints of the experimenters who handled the geckos and confirmed that their NALDI spectra were very different in the region from m/z 600 to 1000. The inside of a freshly molted toe pad shed and the tail of a gecko were also pressed on the NALDI target plate for comparison. The prints generated after a single touch were imaged using a minimal laser spot size (about 5 μm) and a lateral resolution of 50 μm. Three different geckos were used in the NALDI measurements. One individual was used for the cleanly shed spectra, another individual was used for the post-shed spectra and finally a third individual was used for tandem mass spectrometry (MS^2) measurements. In total, four tokay geckos were used for data collection in both spectroscopic techniques. The MS^2 experiments were performed using an ion gate and the LIFT mode; the ion gate is timed to transmit only the desired precursor (all other ions formed by NALDI are removed from the beam path) and the LIFT mode induces dissociation of the selected precursor into structure indicative fragments. Individual mass spectra were analysed using the FlexAnalysis program, and the two-dimensional images of lipids were reconstructed using the FlexAnalysis program (both by Bruker).

2.4. Sum-frequency generation spectroscopy

SFG spectroscopy involves the spatial and temporal overlap of a high-intensity visible laser beam of frequency ω_{vis} with a tunable infrared beam of frequency ω_{IR} to generate SFG beam of frequency $(\omega_{vis} + \omega_{IR})$ [14]. According to the dipole approximation, the generation of SFG photons is forbidden in the bulk and is permitted only at the surface and the interface, where the symmetry is broken. The SFG signal is resonantly enhanced when the frequency of tunable infrared overlaps with the molecular vibrational mode (both infrared- and Raman-active modes). Hence, SFG is sensitive to both the composition and orientation of molecules at the surface/interface. Further enhancement of one- and two-orders of magnitude is achieved when the input beams are incident at angles close to the critical angle for total internal reflection. We used total internal reflection geometry [15] to probe the toe–sapphire interface [16] (figure 3*e*).

SFG spectra were acquired at ambient conditions using a picoseconds laser system (Spectra-Physics) with a tunable infrared beam (2000–3800 cm^{-1}, 1 ps pulse width, 1 kHz repetition rate and a diameter of 100–200 μm) and a visible beam (800 nm, 1 ps pulse width, 1 kHz repetition rate and a diameter of 1 mm). The sapphire prisms were first wiped with toluene using a soft tissue and sonicated in toluene for 1 h. Subsequently, the prisms were washed with deionized water and blow-dried using dry nitrogen. Before each experiment, the sapphire prisms were plasma-cleaned. Six SFG spectra were collected from a single toe obtained from a captive tokay gecko. Contacting interfacial SFG spectra are obtained by pressing a gecko toe against the prism. The gecko adhesive system is directionally dependent and requires a small shear force to align the setae and spatula. To ensure the best possible contact interface for spectroscopy, we replicated the normal and proximal shear force typical of a gecko's natural footfall [1]. Adhesion was confirmed by prolonged attachment of the toe to the sapphire prism. Immediately after the spectra for dry attachment were completed, the gecko toe was detached and the footprint residue on sapphire surface was then measured by SFG. Another clean prism was coated with a thin film of water (by depositing a 3 μl water droplet on a 1.5 cm^2 sapphire surface) and again the toe was attached and SFG spectra were collected to compare the wet and dry conditions. Two input laser beams were incident on the prism face at 42° and 10° with respect to the surface normal for monitoring the sapphire–air and sapphire–gecko toe pad contact interface, respectively. The experiments were performed by scanning the incident tunable infrared beam (from 2750 to 3800 cm^{-1}). The SFG signal is collected using

Figure 1. The invisible and hydrophobic residue of a gecko's step left behind on the substrate. (*a*) Picture of a gecko (*Gekko vittatus*) sticking on a glass substrate. (*b*) After the gecko walked on the clean glass, the glass panel was placed in a room at 98% humidity, allowing moisture to condense on the glass surface. The glass panel became opaque as a result of tiny water droplets covering the surface. The outline of footprints was revealed by differential condensation around the footprints. The picture was collected using a digital camera. Similar footprints were also observed for *Gekko gecko* and *Anolis carolinensis* (electronic supplementary material, figure S10). (Online version in colour.)

a photomultiplier tube after passing through a series of dichroic reflectors, neutral density filter, focusing lens and spectrometer. The spectra were collected using SSP (s-polarized SFG, s-polarized visible and p-polarized infrared beams) and PPP (p-polarized SFG, p-polarized visible and p-polarized infrared beams) polarization combination at room temperature (see electronic supplementary material).

3. RESULTS AND DISCUSSION

The gecko (*Gekko vittatus*) footprint left behind on a glass panel is shown in figure 1. The photograph of gecko footprints in figure 1 was taken after exposing the glass substrate to high humidity. The region where the gecko walked is rendered hydrophobic and water condenses as droplets while the rest of the clean glass substrate wets with a uniform thin layer of water. This provides the contrast to trace the steps of the gecko as it walks on the clean glass. We also observed these footprints on hydrophobic substrates (glass slides coated with fluorinated molecules). The tokay gecko (*Gekko gecko*) and the anole (*Anolis carolinensis*), another adhesive seta-bearing lizard, also showed visible footprints when placed on glass (see electronic supplementary material), presumably of similar material. Because geckos are not known to have secretory glands on their adhesive pads, the presence of hydrophobic residue left behind is puzzling and unexpected.

To characterize the composition of the footprint residue left on the surface, we used the high sensitivity of matrix-free NALDI-MS [17]. The nano-structured NALDI plate triggers the desorption and ionization of the adsorbed analyte molecules for mass analysis. The sample can be transferred directly to a NALDI plate without the need for the matrix deposition process. To determine the spatial organization of the sample, NALDI is capable of mapping the two-dimensional distribution of multiple chemical signatures in one sample. The mass spectrum and mapping image of the footprint were obtained after one gentle contact of a gecko toe with the NALDI plate. To ensure that the mass spectroscopy results were not affected by any contaminants, we conducted the NALDI experiments during the gecko's moulting cycle. In the last phase of cycle, where the old skin is loose and ready to be rubbed off by the gecko, the old skin was manually peeled off the feet and the fresh setae were placed directly into contact with the NALDI plate. In the resulting mass spectrum, shown in figure 2*b*, we observed several ion signals corresponding to phospholipids and sphingolipids, determined by comparing the mass of the ions with the lipid database (see electronic supplementary material).

To confirm the assignments of these ions to lipids, we also conducted MS^2 experiments on the six major peaks observed in the NALDI spectrum. The MS^2 spectrum of m/z 734 is displayed in figure 2*e* (m/z stands for atomic mass/charge). The fragment at m/z 184 contains the phosphocholine head group and the remaining complementary fragment, formed by loss of 183 Da, is observed at m/z 551. The m/z 478 and 496 peaks arise from the loss of one C16 fatty acid molecule (256 Da) and its dehydrated form (238 Da), respectively. On the basis of this fragmentation pattern, the species at m/z 734 is identified as phosphatidylcholine (PC 16 : 0/16 : 0; 1,2-diplamitoyl-*sn*-glycero-3-phophatidylcholine). The MS^2 analysis of the six chemical species showed a common fragment at m/z 184, corresponding to the phosphocholine head group, indicating that they all contain a common head group. The detailed analysis of the other five molecules is provided in the electronic supplementary material.

The spatial distribution of the NALDI ion at m/z 184 (phosphocholine head group) was used to obtain the image plotted in figure 2*a*. The shape of the gecko toe is clearly outlined by the intensity of m/z 184 peak, with the bright intense regions corresponding to higher intensities of the m/z 184 signal. These bright regions also correlate with the regions where the setae-bearing scansors made contact with the substrate. The wear residue is maximum in regions (setae-bearing pads) where adhesion is expected to be the strongest. The comparison of an optical image and the image acquired by NALDI clearly shows that the bright regions in the NALDI spectra are where the setal arrays are located.

To eliminate the possibility that the lipid residue was transferred from the shed skin merely as a part of the shedding process, NALDI-MS and imaging were also performed for a gecko that had shed its skin three weeks prior to our experiments (insets in figure 2*b*,*d*). The spectral pattern of the residue left behind was very similar to the first contact right after shedding. The dominant peaks were still associated with PC

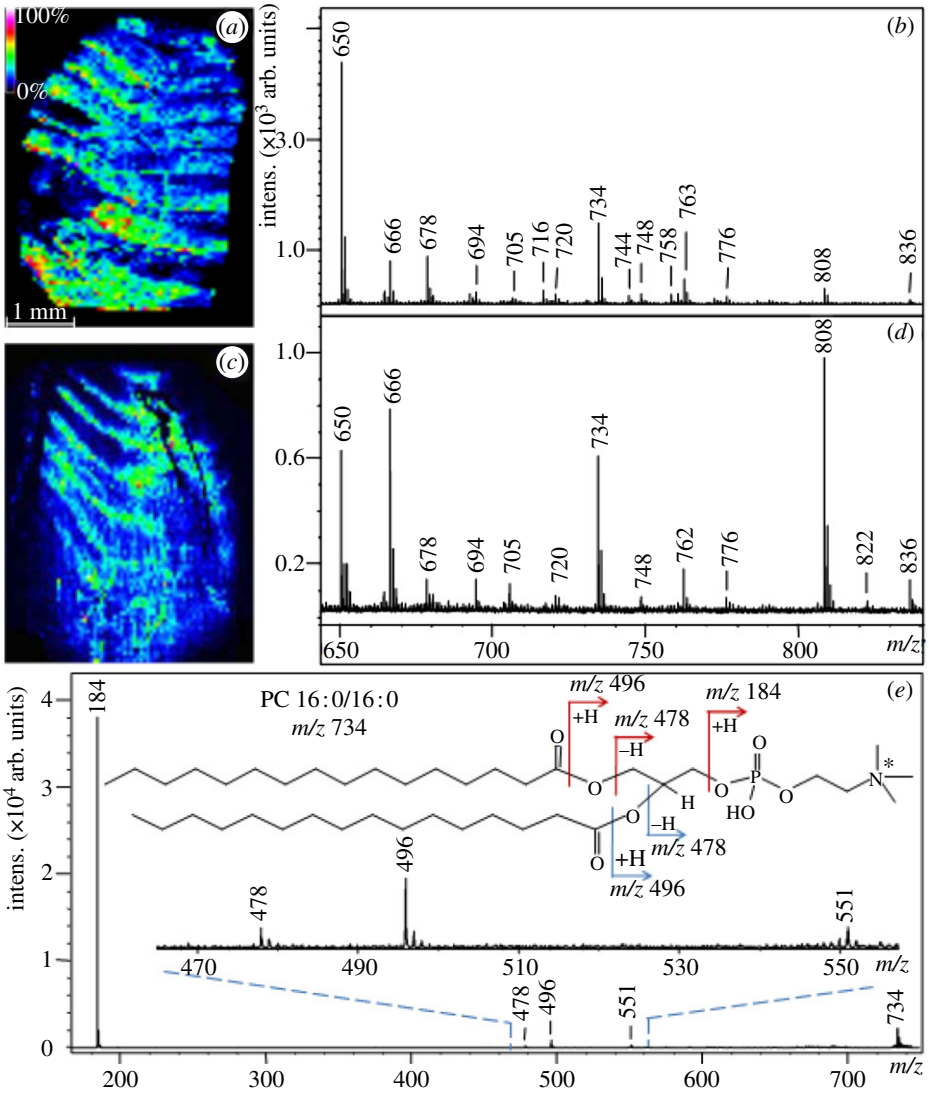

Figure 2. Mass spectroscopy analysis and imaging of the gecko footprint. (*a*) The NALDI mapping image of the peak m/z 184 collected after the gecko toe was brought in contact with the NALDI plate immediately after shedding so that the new setae were exposed. The increased brightness (intensity) in the seta-bearing scansors corresponds to higher concentrations of the substance yielding the m/z 184 signal. (*b*) The scan of the ions observed at m/z 600–1000 in the NALDI spectrum. The spatial distributions of several ions in the NALDI spectrum were mapped and they also show similar images to that obtained for m/z 184 (see electronic supplementary material, figures S2 and S7). For results shown in (*c*,*d*), the geckos were housed for three weeks after their moulting cycle in a glass cage, then their feet were cleaned by touching them many times on a clean glass substrate. The NALDI mass spectrum was collected after this process by touching the gecko toe on the NALDI substrate. (*c*) Mapping of the m/z 184 ion signal, whereas (*d*) ions observed in the range from m/z 600 to 1000. (*e*) Tandem mass spectrum of m/z 734. The major fragment peak at m/z 184 corresponds to phosphocholine head group. The remaining neutral chain (without the head group) appears as a weak peak at m/z 551. Further, the m/z 478 and 496 peaks indicate the loss of one of C16 fatty acids tails from precursor and its dehydrated form, respectively. The fragmentation pattern identifies m/z 734 as phosphatidylcholine 16 : 0/16 : 0.

or sphingomyelin (SM). Similar to the results in figure 2*a*, the footprint image showed that the maximum residue was left behind where the setae-bearing scansors were in contact with the NALDI-MS plate. The strong signal observed, even after three weeks, suggests that the wear of lipids in the form of a gecko footprint is part of its natural cycle of stick and release. The similarity in the NALDI spectra and the mapping images from the fresh and three-week-old gecko footprints also dispels the possibility that these lipid residues originated from residue left behind after the molting process. When a gecko sheds its skin, the old skin along with the shedding complex is removed, leaving fresh setae behind. To test for a relationship between the shed complex and the footprint material, we analysed the NALDI spectrum from the back side of the shed complex which was separated from the newly emerging setae. Interestingly, we observed several peaks in the NALDI spectrum that were characteristic of the lipid residues found on the gecko footprint (spectrum provided in electronic supplementary material, figure S8). This finding points out that the lipid layer on the gecko setae and spatula may potentially play a role in the separation of the shed complex from the emerging setae. We also measured the NALDI spectrum after pressing the non-sticky gecko tail on the surface and observed no measurable NALDI signals at the m/z 184 and in the m/z 600–850 region (see electronic

supplementary material, figure S9). The transfer of the lipid residue after the gecko adhesive pad touches surfaces is a consequence of strong adhesion and consequently wear associated with the peeling process. Gecko footprints were most visible when the animal was forced to walk vertically or pulled along the substrate simulating a strong adhesion event (see electronic supplementary material for images of footprints from animals pulled along a glass substrate). When the non-sticky tail makes contact, there is no adhesion and consequently no measurable transfer of materials onto the surface. Footprint analysis after contacting the adhesive pads to silicon wafers using spectroscopic ellipsometry indicated an average thickness layer of 1–2 nm left behind on the silicon substrate. It is important to note here that the source of the lipid residue and its maintenance during multiple adhesive events are still unclear and requires further detailed investigation.

The observation of lipid molecules in the scansorial region of the toe pads also sheds light on the superhydrophobic nature of the adhesive pads. Surfaces that exhibit stable high water contact angles (greater than 120°) are rough as well as hydrophobic. The gecko's toe pad has a contact angle of 160° [18]. Because adhesion is found to increase as humidity increases, it was conjectured that the surfaces of the gecko setae and spatula were partially hydrophilic [19]. Although the hierarchical structure of the gecko toe pad lends itself to superhydrophobicity, partially hydrophilic hairs would not exhibit a stable superhydrophobicity solely as a consequence of surface roughness. The hierarchical structure of the gecko toe pad in conjunction with hydrophobic surface chemistry is necessary to form a stable superhydrophobic surface. The hydrophobic lipid residue found in the footprints of the gecko has not only implications for the surface chemistry and composition of the setae but also for the superhydrophobic nature of the setae.

Current theoretical models of gecko adhesion consider only β-keratin as the main constituent of the gecko setae and spatula and it is puzzling that we observe such a strong presence of lipid residue left behind in the gecko footprint. It has recently been shown that lipids are present in the cells of the clear layer during maturation of the inner-generation setae as they progress through branching [20]. More generally, the involvement of lipids in the self-assembly of the keratin protein has been postulated by Lillywhite and co-workers [21,22] to explain the organization of keratin in the epidermis of reptiles. The association of lipids and keratin in the epidermis of reptiles and the localization of lipids in the clear layer during setal maturation suggests that it is not unlikely that the mature, exposed setae may also maintain a high association with lipids. Although lipid molecules also play an important role in reducing water loss [20] in the epidermis, lipids are typically found in the deeper layers of the skin, such as the mesos layer [23]. Our finding that lipids appear to be on the outermost surface of the epidermal setal structures may have important implications in controlling the ductility of the setae among other things. To date, there has been no direct evidence to support the presence of phospholipids on the adhesive setae of geckos and all current models of gecko adhesion rely on modelling β-keratin as the contact material for adhesion. Our results also show potential for an additional role of the lipid molecules as a possible sacrificial layer (a much weaker layer that is lost during adhesion) which has implications for controlling the peeling of gecko toes and wear.

To investigate the adhesive contact region of a gecko toe, we took advantage of the surface sensitivity of infrared-visible SFG to study the molecular structure at the contact interface between the gecko toe and a sapphire substrate. SFG is a second-order nonlinear optical technique that gives information on orientation and chemical composition of molecules at buried interfaces without the need to use any specific chemical markers [24]. Figure 3*e* illustrates the geometry used in our experiments [15]. A gecko toe was brought in contact with a dry sapphire substrate. The SFG signals were measured by scanning a tunable infrared laser from 2800 to 3800 cm^{-1} (figure 3*a*). The C–H stretching vibrations associated with hydrocarbon molecules at the interface will appear as resonant-enhanced peaks in the range from 2800 to 3100 cm^{-1}. Whereas O–H stretching vibrations are expected to show broad peaks from 3200 to 3800 cm^{-1} [16]. The flat region from 3200 to 3800 cm^{-1} in figure 3*a* indicates that the interfacial water layer is absent. The peak at 2875 cm^{-1} is assigned to symmetric vibration of the methyl groups, the peak at 2940 cm^{-1} to the methyl Fermi band and the peak at 2850 cm^{-1} to the symmetric methylene groups. The presence of methyl and methylene groups at the contact interface supports the hypothesis that the surface of the gecko setae is hydrophobic. Because methyl groups of alanine and methylene groups of proline and tyrosine have infrared assignments in close proximity of the peaks observed in the SFG spectrum, these hydrophobic signals could be associated with protein molecules. However, if the SFG signals were from protein molecules, we would also have observed peaks between 3000 and 3100 cm^{-1} associated with polar aromatic groups. The absence of aromatic peaks suggests that it is unlikely that the SFG signals originate only from amino acids of β-keratin. The SFG results also suggest that the observed methyl peaks are not associated with the polar PC head groups. If this were the case, then we would have observed a peak in the SFG spectra at 2975 cm^{-1} [25]. This suggests that the contact surface is not dominated by polar head groups and that either the tails or the methylene sides of the phospholipids are facing the surface. Again this is consistent with the hypothesis that the surface of gecko setae is hydrophobic and the adhesive interaction with the surface is primarily owing to van der Waals energies.

We also collected the SFG spectra after peeling the gecko toe off the dry sapphire substrate (figure 3*c*). The SFG spectrum of the footprint residue shows a strong signal from the methylene and methyl groups confirming that the peeling process involves transfer of hydrophobic materials from the toe onto the sapphire substrate. The blank sapphire before contact with the gecko toe had no hydrocarbon signal (figure 3*d*) and confirmed that the residue left behind

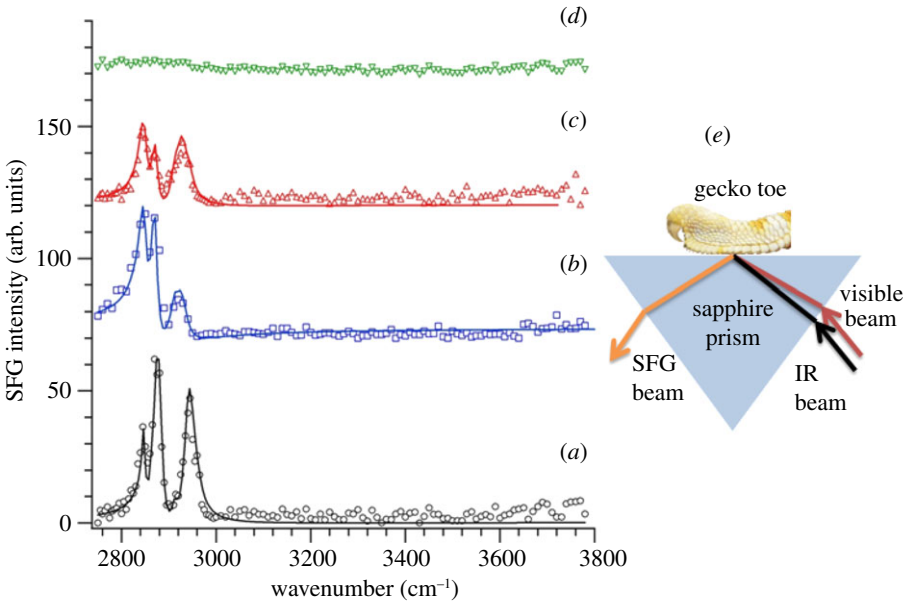

Figure 3. Probing the molecular structure of the contact interface between the gecko toe and the sapphire substrate. (*a*) The SFG spectra collected after bringing the gecko toe in contact with a sapphire prism. (*b*) The sapphire surface was wetted with water and the SFG spectrum was collected after bringing the gecko toe in contact with the sapphire substrate. (*c*) The gecko toe was peeled off the dry substrate and the SFG spectrum was collected of the residue left behind on the sapphire substrate. (*d*) The SFG spectrum of a clean sapphire surface (without any exposure to water) shows no discernible peak in the hydrocarbon and water region. All the SFG spectra are collected using SSP polarization (s-polarized SFG, s-polarized infrared and p-polarized visible beam). Solid lines are fit to a Lorentzian equation described elsewhere [15]. (*e*) Schematic of the SFG experimental measurement. The infrared beam (black line) and the visible beam (red line) were overlapped at the contact interface between toe and sapphire, the SFG beam (orange line) was then analysed. The SFG peaks in the range from 2800 to 3000 cm^{-1} correspond to stretching vibrations of methyl and methylene groups, and the range from 3200 to 3800 cm^{-1} correspond to stretching vibrations of water.

originated from the gecko setae making contact with the substrate.

To understand the influence of water on the adhesion of the gecko toe, we also collected the SFG spectrum after the gecko toe was placed in contact with a wet sapphire substrate. Previous studies revealed that geckos stick stronger to surfaces at low temperature and high humidity [13]. These humidity effects cannot be explained based on van der Waals forces. It has been suggested that there is perhaps a nanometre-thin layer of water between the setae and the surface [11] or that the β-keratin protein rearranges to expose more polar groups on the surface [19]. The SFG technique is sensitive to a monolayer of water, so we tested these hypotheses and even after exposure to water, we did not observe any discernible water peaks between 3200 and 3800 cm^{-1} (figure 3*b*). Interestingly, the methylene peak at 2850 cm^{-1} is much stronger than the methyl peaks after exposing the surface to water. The increase in methylene signal indicates an increase in surface energy of the surface. For example, a well-ordered surface with methyl groups has a surface energy of 20–22 mJ m^{-2}. In comparison, a methylene-covered surface can increase the surface energy to 25–30 mJ m^{-2} depending on the concentration of the methylene groups at the surface [26]. However, the presence of methyl and methylene groups, even after exposure to water, indicates that the gecko adhesive pads before and after exposure to water continue to be hydrophobic and efficient in repelling water.

Based on our SFG and NALDI mass data and high-resolution electron microscope images of the gecko setae and spatula by Rizzo *et al.* [9], we propose two tentative models for the contact interface of the gecko adhesive system. In the first model, a homogeneous layer of lipid molecules covers the keratin of the spatulae and comes in direct contact with the surface (figure 4*a*). This lipid layer breaks up during the peeling process and leaves a trace of the residue as a gecko footprint. The second model is heterogeneous, where there is a combination of lipid molecules between the keratin rods or inter-dispersed in the spatular pad (figure 4*b*,*c*). The residue left behind could be a combination of parts of keratin protein as well as lipid molecules. Our observation of discrete peaks in the NALDI spectrum indicates it is unlikely that we have residue in the form of small keratin proteins because this would have resulted in a cluster of peaks in close vicinity with each other. Regardless of homogeneous or heterogeneous lipid distribution at the interface, current contact models of the gecko adhesive system need to be corrected. Our model suggests that the sticky toe pads of geckos can no longer be analysed or modelled as a completely homogeneous protein-based fibrillar adhesive system.

The observations of an increase in shear adhesion at high humidity observed in single seta and animal experiments have also been puzzling [11,13]. There have been several mechanisms proposed in the literature to explain the humidity effects. It has been suggested that an

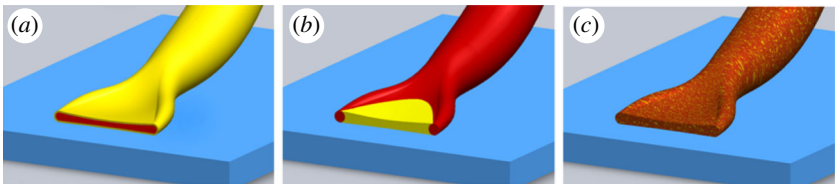

Figure 4. Models visualizing the location of lipid molecules on the spatula. In the homogeneous model (*a*), lipid material (yellow) may form an evenly distributed layer encasing the spatular structure. This would be equivalent to a lipid membrane covering keratin fibres (red). However, our results cannot exclude a heterogeneous model for contact (*b*) and (*c*), where the lipid material is not responsible for all the contact at the interface, allowing either lipid material or keratin protein to make contact with the solid substrate. Within the heterogeneous model there are two possibilities. In (*b*) the lipid molecules form the inter-layer between the two keratin rods or in (*c*) the lipid material is evenly distributed with the keratin proteins.

increase in normal adhesion is associated with capillary forces [12]. However, single setae adhesion measurements by Huber *et al.* [11] concluded that the increase in adhesion cannot be owing to capillary bridges. Pesika *et al.* [19] suggested that protein restructuring may be responsible for increased adhesion, although no direct evidence was presented in that work. Recently, Autumn and co-workers [27] suggested that the humidity effects on shear and normal adhesion could be explained based on changes in mechanical properties of setae. It is important to recognize that the effects of humidity on adhesion are not consistent across different studies and further work may be necessary to completely understand the response of this system in humid environments. Our contribution to this debate is that our experiments clearly indicate that water is not observed at the contact interface, directly refuting the hypothesis that a nanometre-thin layer of water is responsible for increasing normal or shear adhesion in high humidity. We also observed an increase in methylene concentration at the surface after exposure to water implying restructuring of the surface groups does occur. Because shear adhesion depends on both modulus and surface energy, changes in both these quantities as a function of humidity will affect the shear forces. Here, the observation of wear and the presence of phospholipid residue on the surface introduces additional complexity to the system which may also influence the shear adhesion as a function of humidity.

The observation of the wear in setae-bearing regions of the gecko toe pad raises the question of whether a gecko's ability to stick to surfaces is compromised with time. It is possible, through a variety of mechanisms, that the setae are worn out with time. As a result of the natural shedding process, geckos replace setae approximately once a month (A. Stark 2011, personal observation). On shorter timescales relevant to the organism (i.e. between shedding cycles), we have not observed systematic changes in adhesion over time which lead us to suspect that adhesion is not compromised by each footstep an individual takes. For example, we have measured the adhesive strength of many species of geckos at many different times during their shedding cycle and we have not observed a correlation between the stage of shedding cycle and variation in adhesion, nor has this been reported in the literature. Therefore, we believe that the effects of wear will be gradual over time and may be of little consequence to the overall adhesion of the organism at relevant timescales. Instead, the presence of lipids and lipid residue may be more important in advancing our understanding of the mechanisms employed by geckos to adhere to various substrates in different conditions.

4. SUMMARY

In summary, we have shown that geckos deposit lipid molecules on the surfaces they walk across. Although geckos do not use glue for adhesion, a fibrillar adhesive system made of other materials in addition to β-keratin raises several intriguing possibilities. The presence of phospholipid molecules left behind as wear residue may be important in fluid-like adhesion and release of the fibrillar hairs. Thus, the presence of the sacrificial lipid layer might protect the degradation of the β-keratin spatulae. The hydrophobicity of the hydrocarbon tail of lipid molecules exposed on the surface explains the resilience of these materials to exhibit self-cleaning superhydrophobic properties. Most importantly, our model suggests that the sticky toe pads of geckos should no longer be analysed or modelled as a completely homogeneous protein-based fibrillar adhesive system. The finding of lipid materials on geckos' adhesive pads also has important implications for material scientists trying to design synthetic structures that could be used thousands of times, similar to geckos.

All procedures involving live animals were approved by the University of Akron IACUC protocol 07-4G, and were consistent with guidelines published by the Society for the Study of Amphibians and Reptiles (SSAR 2004).

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A.Y.S. photographed the footprint. P.Y.H, X.L. and C.W. designed and performed the NALDI imaging and tandem mass spectrometry experiments and helped in interpreting the results. P.Y.H. and A.D. wrote the paper. A.Y.S., P.H.N., C.W. and X.L. assisted in writing the manuscript. The authors declare that they have no competing financial interests.

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