Evidence of a molecular boundary lubricant at snakeskin surfaces

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During slithering locomotion the ventral scales at a snake’s belly are in direct mechanical interaction with the environment, while the dorsal scales provide optical camouflage and thermoregulation. Recent work has demonstrated that compared to dorsal scales, ventral scales provide improved lubrication and wear protection. While biomechanic adaption of snake motion is of growing interest in the fields of material science and robotics, the mechanism for how ventral scales influence the friction between the snake and substrate, at the molecular level, is unknown. In this study, we characterize the outermost surface of snake scales using sum frequency generation (SFG) spectra and near-edge X-ray absorption fine structure (NEXAFS) images collected from recently shed California kingsnake (\textit{Lampropeltis californiae}) epidermis. SFG’s nonlinear optical selection rules provide information about the outermost surface of materials; NEXAFS takes advantage of the shallow escape depth of the electrons to probe the molecular structure of surfaces. Our analysis of the data revealed the existence of a previously unknown lipid coating on both the ventral and dorsal scales. Additionally, the molecular structure of this lipid coating closely aligns to the biological function: lipids on ventral scales form a highly ordered layer which provides both lubrication and wear protection at the snake’s ventral surface.

1. Introduction

Snake skin is a highly functional biological material. General epidermal functions include displaying colours, sensory perception, thermoregulation, [1] and barrier against water loss [2] and radiation [1,3]. The slithering locomotion of snakes requires additional frictional properties [4] and resistance against wear [5]. The peculiar locomotion of snakes has inspired engineers for years. For example, robotics researchers are trying to mimic the snake’s capability to negotiate a range of challenging terrain types [6]. Research conducted over the last century reveals functional adaptation of both the inner architecture (micron scale) and surface morphology (nanometre scale) of snake skin. The epidermis of snakes is a multi-layered system consisting of keratin and associated $\beta$-proteins [7] that can be histologically distinguished from top to bottom into the mesos-, the $\alpha$-, the lacunar- and the clear layer, covered by an outer shell of two hard layers of $\beta$-keratin—the exterior Oberhäute and the $\beta$-layer [5,8,9]. In many snake species [10–12] the Oberhäute features specific morphologies—the so-called microornamentation of the scale [13,14]. Several studies have demonstrated the relevance of these micro- and nanostructures on dorsal and ventral scales for frictional properties, optical appearance, self-cleaning and water-repellency [4,15–17]. However, if we ever hope to mimic the optical and mechanical properties that snake scales possess, we must first define the chemical environment of the Oberhäute.
Detailed studies of the biochemistry and morphology of the snake epidermis have been carried out. X-ray diffraction studies have identified the two types of keratin in snake skin (α- and β-layers) [18] and have also provided insight into the orientation of protein fibres within these epidermal regions [19]. A later study focusing only on ventral snake scales, in two species (Notoechis scutatus and Bitis gabonica), investigated not only the formation of keratins and associated β-proteins, but also that of lipids within the keratin layers [20]. Three skin layers, differing in their content and molecular assembly of proteins and lipids could be distinguished [20]. X-ray diffraction also suggested that the lipids in the mesos-layer are organized in a lamellar fashion and have aliphatic chains oriented perpendicular to the scale surface. These studies showed that in both species the outer layers of the ventral scales contained both proteins, with β-sheet conformation, and lipids [20]. Immunocyto-chemical studies of the biological composition of skin of colubrid (Pantherophis guttatus and Natrix natrix) and viperid snake species (Crotalus atrox and Bitis gabonica) consistently revealed glycine-cysteine-rich corneous β-proteins accumulated in the cells of the Oberhautchen of the snake skin, which decrease in the underlying β- and α-layers [21–23].

Crucial to the mechanical properties of the snake skin, is not only the chemical composition and the spatial distribution of keratins, associated β-proteins and lipids within the epidermis but also the nanoscale chemistry of the outermost epidermal surface [4,5]. Much to our surprise, the molecular structure of the epidermis, which is the most relevant region for external influences, has not yet been investigated adequately. Here, we identify the biochemical composition and structure of the outer surface of ventral and dorsal scales taken from Lampropeltis californiae. Combined, this study demonstrates that the β-layer—the Oberhautchen—the outermost layer in contact with the environment, is in fact covered with a nanometre thin, lipid film with a structure that is correlated with the biological function of snake epidermis.

2. Results and discussion
To initially characterize the chemistry at the scale surface, we collected near-edge X-ray absorption fine structure (NEXAFS) microscopy images from both L. californiae shed dorsal and ventral scales. NEXAFS probes the chemical state, orientation and structure of species within the outer 5 nm of a material surface [24]. NEXAFS has been developed to provide a detailed analysis of biological surfaces and has been used to track the binding, orientation and structure of small molecules, proteins and DNA when attached to surfaces [25–30].

In the resulting NEXAFS images shown in figures 1–4, each pixel contains an entire NEXAFS spectrum, [31–33] thus, providing an examination of the ordering of molecular bonds at the scale surfaces while in parallel allowing us to map chemical species across the surface. At the specific electron retardation voltage used in our NEXAFS experiments (50 eV), we expect the NEXAFS sampling depth to be approximately 4 nm [33]. Pre- and post-edge normalized NEXAFS images of the dorsal and ventral scales can be found in figures 1–4.

Mapping the relative intensities of different spectral regions at the C K-edge provides a detailed characterization of the molecular environment across the scales. C K-edge NEXAFS spectra, exported from a region of interest are presented in the right panel of figures 1 and 2. The pre-edge π* resonance at 285.3 eV representative of C=C bonds is present in all spectra taken from both types of scales [24,34]. Moving down the spectra to higher X-ray energies, we observe a shoulder at 287.4 and a broad resonance at 294.0 eV related to R*/C−H σ* and C−C σ*, respectively. There is also a sharp π* resonance at 289.0 eV—related to

![Image of NEXAFS C K-edge ventral scale results](https://example.com/image.png)

**Figure 1.** NEXAFS C K-edge ventral scale results. Panels from left to right: exported C K-edge NEXAFS spectrum at X-ray incidence angles of 70° and 30°; pre and post-edge NEXAFS images (at 30°) related to different spectral regions at the C K-edge: C=C π*, σ* C−H and C=O π* bonds. In all images, the head of the snake (rostrad) is to the left and the tail (caudad) is to the right. (Online version in colour.)
C=O bonds. N K-edge spectra of the scales are presented in the right panel of figures 3 and 4. Each spectrum is dominated by a broad $\sigma^*$ resonance near 405.5 eV, attributed to N–C bonds, and an intense $\pi^*$ transition at 399.0 eV assigned to amide groups [25,27,29].

The orientation and ordering of molecular bonds can be determined by simply following any change in the X-ray absorption as we change the angle of the sample normal with respect to the incident X-rays. Difference spectra (70°–30°), extracted from identical regions from the ventral and dorsal scales can be found in figures 1 and 2, respectively. Both types of scales exhibit a large positive dichroism at the C=C $\pi^*$, $\sigma^*$ (C–H) and the $\pi^*$ resonance related to C=O bonds. A comparison of the two difference spectra demonstrates a higher degree of ordering (larger positive dichroism) of the $\sigma^*$ (C–H) shoulder and the C=O $\pi^*$ resonance for the ventral scales than the dorsal. However, these ordered C–H, C=C and C=O bonds can stem from both proteins and lipids, and may play an important role in the lubrication and wear protection of the ventral scales as they come into contact with surfaces.

Closer examination of the difference between the 30° and 70° nitrogen K-edge spectra (right panel of figure 3), reveals a negative dichroism for the resonance at 399.0 eV, from the spectrum extracted from the ventral scales. This observed dichroism relating to the N 1s transition to the $\pi^*$ orbital of...
the peptide bond (399.0 eV) can originate from a range of protein secondary structures. However, helical structures are not expected to contribute significantly to the angle dependence of amide peaks due to the broad distribution of orientations within the helix [29]. Hence, the majority of the observed negative dichroism stems from peptide bonds present within β-sheet structures. This corroborates previous work that reported a cuticle layer composed of ordered fibrils of β-protein (β-keratin) [20].

The relative intensities of specific molecular bonds at the C and N K-edge are mapped in figures 3–6. The images demonstrate that the chemistry across the dorsal scale surface is relatively uniform. However, highlighting the intensities of the region of interest over which the spectra in the left panel were averaged is highlighted. In all images the head of the snake (rostrad) is below and the tail (caudad) is above. (Online version in colour.)

**Figure 4.** NEXAFS N K-edge dorsal scale results. Panels from left to right: exported N K-edge NEXAFS spectrum at X-ray incidence angles of 70° and 30°; pre- and post-edge NEXAFS images (at 30°) related to different spectral regions at the N K-edge: amide π* and σ* N–C bonds. The region of interest over which the spectra in the left panel were averaged is highlighted. In all images the head of the snake (rostrad) is below and the tail (caudad) is above. (Online version in colour.)
Oberhäutchen layer of the scale and a few nanometres into the β-layer. Yet, the large positive dichroism observed in the carbon K-edge NEXAFS spectra, from both scales, still corroborate the SFG results by confirming a large degree of ordering of C=C, C–H and C=O bonds. Combined the SFG and NEXAFS results demonstrate the presence of a layer of lipids at both the ventral and dorsal scale surfaces. It is unclear at this point how the lipid layers are stabilized.

**Figure 5.** (a) SFG spectra amide I (1650 – 1850 cm\(^{-1}\)) of ventral (top) and dorsal (bottom) scales. Both spectra were acquired at PPP polarizations. The single peak centred at 1746 cm\(^{-1}\), found in both spectra, is characteristic of ordered ester –C–(C=O)O – bonds. (a) SFG spectra collected at the C–H stretching region (2700 – 3100 cm\(^{-1}\)) collected from ventral (top) and dorsal (bottom) scales. Both spectra were acquired at PPP polarization. The dorsal scale contains two symmetric methylene resonances at 2850 and 2865 cm\(^{-1}\). The peak at 2865 cm\(^{-1}\) is a signature of a methylene mode adjacent to a methyl group. The spectrum taken from the ventral scale contains three peaks: a peak at 2850 cm\(^{-1}\), which is again related to symmetrical methylene stretches, and two additional peaks at 2875 and 2975 cm\(^{-1}\), which are associated with CH\(_3\) symmetric and antisymmetric modes, respectively. (Online version in colour.)

**Figure 6.** Diagram of the lipid layer on the ventral and dorsal scale surfaces. The surface chemistries of the ventral and dorsal scales are unique. An ordered ‘solid like’ lipid layer is present at the ventral scale versus a semi-disordered lipid layer on the dorsal surface. The terminology follows [35].

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and maintained at the outer surface. One possible scenario would be that the lipids are effectively anchored to cystein-rich β-proteins of the β-layer via disulfide bonds similar to mammalian corneous layers [52,53]. Another possibility is that the lipid layers are regenerated by diffusion of lipids, through the fibrous β-layer, which impregnate reptilian skin to prevent water evaporation [54]. However, the exact mechanism for lipid regeneration is still unknown and needs to be characterized further.

One important difference observed between the SFG spectra taken from the two types of scales is the presence of vibrational modes associated with ordered CH₃ groups at the ventral scale surface. The CH₃ groups likely stem from the terminal methyl group at the end of the surface bound lipids and the presence of methyl modes only within the ventral spectra suggests that the degree of lipid ordering at the ventral surface is higher than at the dorsal surface (figure 6) [49].

While the dorsal and ventral ornamentations at the micro-metre level may not differ much (electronic supplementary material, figures S1 and S2), the surface chemistries of the ventral and dorsal scales are unique. An ordered ‘solid-like’ lipid layer is present at the ventral scale (figure 6) versus a semi-disordered lipid layer on the dorsal surface. Previous measurement of the frictional properties of dorsal and ventral scales from other species report large differences between the ventral and dorsal scales [4,5,55,56]. Ventral scales possess a lower friction coefficient compared with dorsal scales [5,35]. This reduction of friction at the ventral surface could be a direct result of the observed well-ordered lipid monolayer acting as a lubricating layer [57]. In fact, studies of friction between vertebrate joints have revealed that adsorbed monolayers of synovial surfactants act as boundary lubricants reducing the coefficient of kinetic friction between joints [58]. In addition, lipids extracted from synovial fluid exhibit excellent anti-wear properties [59,60]. This all suggests that the ordered lipid layer observed at the ventral surface has been optimized for both friction and wear reduction.

In summary, this multispectroscopic study reveals the presence of a nanometre thin lipid layer at the outermost surface of L. californiae scales. The lipids structure and alkyl chain alignment differ markedly between ventral and dorsal scales: the film at the ventral scales exhibits a higher degree of order and denser packing compared with dorsal scales. Ventral scales are in contact with the environment, and high lipid film order, which improves lubrication and reduces wear, will improve mechanical contact with the environment during slithering locomotion.

3. Experimental methods

Ventral and dorsal scale samples were taken from moult skins of three adult individuals of L. californiae and kept under argon until analysis [61]. Care was taken to collect data from the outside surface of the scales. Since L. californiae sheds its skin inside out, the side pointing in was probed. The samples were visually inspected for contamination and beam damage before and after the experiments. Multiple spectra were measured at each spot. The spectra did not change over time, which demonstrates that beam damage did not change the data significantly. Samples from three different individuals were included in the study and the collected spectra were identical.

The animals were obtained by a private breeder and kept in a terrarium on conventional litter, through several moult cycles. The scales were mounted on a flat non-reflective substrate with double-sided tape with the outside skin surface pointing away from the surface.

SFG vibrational spectroscopy spectra were obtained by overlapping, in time and space, visible and IR pulses of light. The visible beam, centred at 800 nm (pulse duration and energy of 35 fs and 4 mJ, respectively) was delivered by a regenerative amplified titanium–sapphire system (Spitfire Ace, Spectra Physics, Inc) and then spectrally narrowed (approx. 15 cm⁻¹) using a Fabry–Perot etalon. IR pulses were generated by splitting the amplified visible beam and using it to pump an optical parametric amplifier coupled to a collinear difference-frequency generator (TOPAS, Light Conversion). This IR beam then passes through a half wave plate polarizer before being focused onto the sample surface. All SFG spectra were collected at the ppp polarization combination (p-polarized SFG signal, p-polarized input visible and p-polarized input IR beam at the sample). The SFG signal was then focused into a spectrograph (Acton, Princeton Instruments) where it was dispersed by a grating and focused onto an electron multiplied CCD camera (Newton, Andor). All spectra were normalized by the IR beam profile. Spectra were collected from multiple scales taken from three different individuals.

Carbon and nitrogen NEXAFS microscopy images were collected on the parallel processing imaging system at the NIST U7A beamline at the National Synchrotron Light Source (NSLS—Brookhaven National Laboratory) A soft X-ray beam, with energy scanned around the carbon K-edge (270–340 eV; resolution approx. 0.1 eV; flux approx. 5 × 10¹⁰ photons s⁻¹ (0.1% BW)), was rastered across an 18 × 13 mm² area on the sample. A large area rapid imaging analytical tool (LARIAT, Synchrotron Research Inc.) was used to measure partial electron yield. Step size for the carbon K-edge scans was 0.1 eV with a 2-s dwell time. Nitrogen K-edge data were collected around 390–430 eV with a resolution of approximately 0.2 eV. The step size for the nitrogen K-edge scans was 0.2 eV with a 3-s dwell time. The emitted photoelectrons were guided to an electron yield detector by a full field imaging parallel magnetic field. This produced a series of two-dimensional NEXAFS images with a 50 μm spatial resolution. To eliminate the effect of incident beam intensity fluctuations and absorption features in the beamline optics, the PEY signals were normalized by the photo yield of a clean gold slit frame located upstream along the path of the incident X-ray beam. This NEXAFS imaging endstation can handle a range of conducting and insulating samples without charging effects [31–33]. All images presented here have been pre- and post-edge normalized.

Data accessibility. Datasets are available upon request by email.

Author contributions. J.E.B., M.S., S.N.G. and T.W. designed the experiments. J.E.B., C.J., D.A.F. and T.W. conducted the SFG and NEXAFS experiments. The manuscript was written by all co-authors.

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