Mineralized soft-tissue structure and chemistry in a mummified hadrosaur from the Hell Creek Formation, North Dakota (USA)

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An extremely well-preserved dinosaur (Cf. Edmontosaurus sp.) found in the Hell Creek Formation (Upper Cretaceous, North Dakota) retains soft-tissue replacement structures and associated organic compounds. Mineral cements precipitated in the skin apparently follow original cell boundaries, partially preserving epidermis microstructure. Infrared and electron microprobe images of ossified tendon clearly show preserved mineral zonation, with silica and trapped carbon dioxide forming thin linings on Haversian canals within apatite. Furthermore, Fourier transform infrared spectroscopy (FTIR) of materials recovered from the skin and terminal ungual phalanx suggests the presence of compounds containing amide groups. Amino acid composition analyses of the mineralized skin envelope clearly differ from the surrounding matrix; however, intact proteins could not be obtained using protein mass spectrometry. The presence of endogenously derived organics from the skin was further demonstrated by pyrolysis gas chromatography mass spectrometry (Py-GCMS), indicating survival and presence of macromolecules that were in part aliphatic (see the electronic supplementary material).

Keywords: hadrosaur; Hell Creek; soft tissue

1. INTRODUCTION

The recognition of dinosaur soft-tissue structures and organic molecules preserved inside bone has been previously reported in a number of publications (see Schweitzer et al. 2007 for review). The presence of both organic structures and molecules in dinosaur soft tissues such as skin, terminal ungual phalanx sheath, tendon or degraded collagen fibres has been only rarely reported (Schweitzer et al. 1999a,b; Lingham-Soliar et al. 2007). Here we report the preservation of soft-tissue replacement structures and the presence of organic molecules associated with a hadrosaur dinosaur, Edmontosaurus sp. (MRF-03), from the Upper Cretaceous, Hell Creek Formation of North Dakota, USA. Large areas of uncollapsed skin ‘envelope’ are preserved through early mineralization around much of the fossil including the tail, legs and an arm. Skin impressions observed on most other dinosaur ‘mummies’ are predominantly interpreted as trace fossils (Sternberg 1953; Martill 1991; Kellner 1996; Murphy et al. 2002); however the integument of MRF-03 displays both depth and structure (figures 1 and 2).

The depositional environment aided the rapid precipitation of minerals around and within the skin replacing the hide. Similar mineralization of dinosaur skin has been noted from the lacustrine Las Hoya limestone (Briggs et al. 1997) and a marine carbonate mudstone (Martill et al. 2000). Structures consistent with melanosomes have also been imaged in fossil feathers from the marine Crato Formation (Early Cretaceous) of Brazil (Vinther et al. 2008), although previous studies

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suggested that similar features were microbial in origin (Wuttke 1983; Bingham et al. 2008). In comparison, MRF-03 is preserved in a terrestrial, probably waterlogged, setting. There has also been mineral replacement of the epidermal laminae of the keratinous sheaths on the terminal ungual phalanx of several digits, indicating that mineralization outpaced the decomposition of these structures. Structural biomaterials present would be expected to fall within what is predicted for the extant phylogenetic bracket (EPB) of crocodilia and Aves (Witmer 1995), constraining the beta-keratin composition for any dinosaur ‘keratinous’ structure analysed (Manning et al. 2006).

The remains of hadrosaur MRF-03 were preserved through rapid burial on the margins of a sandy river channel. Sediment enclosing the specimen is mainly composed of fine sand-sized grains of quartz, feldspars and rock fragments with some higher plant-derived organic matter (Johnson 2002; Fricke & Pearson 2008). Isotopic and chemical analysis of sedimentary cement gives insight into the environment of preservation. Stable isotopic compositions of carbonate cements (CaCO$_3$ and FeCO$_3$) identified by optical microscopy, electron microprobe and SEM; $\delta^{13}$C range 1.58–7.08 and $\delta^{18}$O range −5.13 to −6.67 both per mil relative to pec de belemnite (PDB)) in concretions associated with MRF-03 were obtained. The upper portion of the skin in one thin-sectioned sample clearly showed parallel structures (figure S1, electronic supplementary material (ESM)) that might be expected for the stratum corneum of a vertebrate skin section (figure 2a), comparable with extant vertebrates (Matoltsy 1986).

In order to further map the fine structure of the skin, Environmental Scanning Electron Microscope (ESEM) images of an uncoated thin section of skin from MRF-03 were obtained. The upper portion of the skin in one thin-sectioned sample clearly showed parallel structures that might be expected for the stratum corneum of a vertebrate skin section (figure 2a), comparable with extant vertebrates (Matoltsy 1986).

An electron microprobe was used to map the chemistry of the sectioned skin of MRF-03. The calcium abundance map (figure 3) clearly shows a cell-like texture within and constrained by the skin, revealing two distinct regions. The interior (lower) region (1.5–2.5 mm thick) has low calcium content, compared with the exterior (upper) surface (approx. 1 mm thick). We interpret this as early carbonate growth preserving the original tissue texture of the dinosaur skin. Precipitate texture (approx. 20–30 µm lateral width) and overall cross-sectional thickness of the postulated ‘skin’ are comparable to the cell texture and skin thickness of extant organisms (Bada et al. 1973). Cathodoluminescence imaging of a similar region, completed independently at the University of Liverpool, shows similar structures (figure S1, electronic supplementary material).
supplementary material). Incomplete preservation is possible; however, these observations constrain the minimum thickness of the remaining epidermis for MRF-03.

An iron carbonate matrix that persisted on the distal toes of each pes was reminiscent of organic, sheath-like structures that would have enclosed the bone of each terminal ungual phalanx. The composition of such structures in extant vertebrates is conservative and typically composed of keratin. There are two main types of keratin: alpha and beta. The EPB of dinosaurs (Witmer 1995) suggests beta-keratin would be the structural form produced by this group of animals. Given that beta-keratin is a relatively robust structural protein, we initially focused our organic analysis here. The surface of the iron-rich carbonate that formed the matrix for this structure showed clear signs of breakdown, indicating that the original surface of the terminal ungual phalanx sheath was not present and that a degraded subsurface layer within the keratinous sheath remained.

To avoid contamination, grains (less than 1 mm) were removed from the terminal ungual phalanx sheath matrix and analysed with no further preparation. Stingray FTIR
mapping showed an organic coating on many of the grains (figure 4) that contained an absorption band corresponding to the characteristic amide I band (approx. 1650 cm$^{-1}$). Mapped absorption at the amide II band position (approx. 1520 cm$^{-1}$) showed nearly identical zonation. This FTIR result was repeated on 13 samples from the structure coating the terminal ungual phalanx, suggested the presence of remnant organic molecules. The position and appearance of the FTIR bands of the amide I and II groups present in the terminal ungual phalanx region are directly comparable to the beta-keratin samples taken from pigeon down and crocodile terminal ungual phalanx (figure 4).

(c) Amino acid analyses
Amino acid composition and racemization analyses were carried out as potential screening methods to identify samples suitable for protein analysis. The relative amino acid concentrations for the majority of samples collected from MRF-03, including fragments of the keratinous sheath and skin, were similar to those observed in the surrounding matrix and woody material, but at concentrations greater by nearly an order of magnitude, possibly indicative of microbial contamination (figure 5a). The amino acid racemization results also indicated the presence of microbial contamination with higher D/L values (figure 5b) in slow racemizing amino acids (such as alanine) than in faster racemizing amino acids (such as aspartic acid and asparagines) (Bada et al. 1973). The presence of microbial biofilms may be misleading when attempting to determine the presence of endogenous organics (Kaye et al. 2008). However, the skin envelope sample taken from the base of the tail does exhibit a distinct composition potentially indicative of fibrous structural proteins such as collagens and keratins. Given these amino acid compositions, the skin envelope was then pursued as the region most likely to contain endogenous beta-keratin and was further analysed by various proteomics-based techniques.

(d) Proteomics analyses
A method that successfully separated out 12 kDa beta-keratin proteins from barnacle goose claw using a size exclusion and PAGE protocol was developed at the Wolfson Molecular Imaging Center (WMIC), but when applied to samples taken from the terminal ungual phalanx sheath and skin regions it was unsuccessful at recovering intact proteins. When the size exclusion
step was removed from the preparation protocol, low-
molecular-weight fractions (less than 12 kDa) were clearly
observed on the electrophoresis gel for samples from the
sheath covering the terminal ungual phalanx region and
from the skin, thus indicating the presence of organic
material. Low-molecular-weight fractions were not observed by
this technique in any of the sediment controls taken near the fossil.

Independent analyses at the University of Manchester
School of Life Sciences using matrix assisted laser desorp-
tion/ionization-mass spectrometry (MALDI-MS) and
liquid chromatography-electrospray ionization (LC-ESI)
following various protocols (see electronic supplementary
material) were consistent with the results produced by the
WMIC in that low-molecular-weight peaks at
m/z 1100–2200 were observed. However, the identity of these could
not be ascertained owing to the very low signal and poor
quality MS/MS spectra obtained, clearly a fundamental
issue when dealing with such ancient specimens. Some
identical peaks were determined by this method in the
sediment samples, which further indicates the necessity
to obtain unambiguous MS/MS spectra before making
claims to the identification of endogenous beta-keratin,
except for the promising amino acid composition results.

(c) Pyrolysis of skin
However, the presence of organic compounds specifically
associated with the skin envelope was further demon-
strated by Py-GCMS (figure 6). The pyrolysates reveal a
substantial difference in the aliphatic polymer from
MRF-03 skin samples when compared with the
associated sediment. Py-GCMS of the skin generated
n-alkanes/n-alken-1-ene homologues ranging in carbon
number from C0 to C16 with a trimodal distribution of
n-alkanes (figure 6a; maxima C11, C15 and C22). In
comparison, the n-alkane/n-alken-1-ene homologue
distribution pattern in the enclosing sediment differs consi-
derably, ranging from C0 to C30 but dominated by the
C10–C18 n-alkanes with a maximum at C11 (figure 6b).
The observed differences are inconsistent with an origin
solely via migration from enclosing sediment and thus
must have been derived endogenously. This suggests that
the organics present in the skin envelope include a
macromolecule that is in part aliphatic. Comparable to
earlier studies on plant and insect fossils, these aliphatic
components are interpreted to be the result of a process
of in situ polymerization of original organic compounds
derived from the hadrosaur (Gupta & Pancost 2004;
Gupta et al. 2006, 2007a,b).

(f) X-ray diffraction of terminal ungual phalanx
XRD of samples taken from the terminal ungual phalanx
sheath region was also completed, but this did not show
distinct diffraction peaks corresponding to beta-keratin,
corroborating the conclusion from the proteomic analysis
that any original beta-keratin is probably severely
degraded or extremely dilute. However, a broad back-
ground was observed, consistent with the presence of a
large percentage of organic matter being contained in
this region (along with quartz and feldspar). In contrast,
XRD of sediment samples taken from in and around
MRF-03 did not show such a background, consistent
with the fact that Py-GCMS showed that the macromol-
ecular composition of the sediment was significantly
different from that observed in the skin (figure 6).

(g) Combined analyses of tendon
FTIR, XRD and electron microprobe analyses were also
conducted upon a polished thin section of tendon (trans-
versospinalis) (Organ 2006) recovered from a neural
spine in the caudal region of MRF-03. XRD showed
that the tendon is dominantly composed of the
ossifying mineral apatite (bio-apatite: Ca5(PO4)3(OH)2−x−y
Na+,x−y; maxima C11, C15, C27). In
comparison, the
X-ray diffraction of terminal ungual phalanx
sheath region was also completed, but this did not show
distinct diffraction peaks corresponding to beta-keratin,
corroborating the conclusion from the proteomic analysis
that any original beta-keratin is probably severely
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canals within ossified collagen bundles. The mapped peak corresponds to the Si-O asymmetric stretch of polymerized silica (1000–1300 cm$^{-1}$). Selective mapping (figure 7b) of the carbon dioxide absorption band (approx. 2350 cm$^{-1}$) also shows that carbon dioxide has been trapped in the lining of the Haversian canals (Adams & Organ 2005). Structures within the Haversian canals also become visible via mapping of a prominent absorption band at approximately 1770 cm$^{-1}$ (figure 7c). Definitive assignment of this band is also not possible without further chemical information, although the band position implies the presence of a C=O group. This band is not present in any of the other regions sampled either within or around the fossil or within any of the preparation materials. This suggests that it may be associated with breakdown products of the original organic material deposited within the tendon, consistent with the presence of endogenous organic material identified from other regions of the specimen.

A longitudinal section of the ossified epaxial tendon (figure S3, electronic supplementary material) shows that the incorporation of carbon dioxide is indeed associated with the Haversian canals, but is not uniform along the length of the structure, rather it is discontinuously spread over the surface of the canal. Concentric zonation in cross section of another Haversian canal is shown strikingly via a total reflectance infrared map (figure S4, electronic supplementary material). This indicates compositional heterogeneity within the canal itself. Along with the clear diffraction peaks corresponding to apatite, XRD of the tendon also showed a broad high-intensity background similar to that observed from the skin, which further corroborates the presence of organic material as inferred from the FTIR analysis.

4. CONCLUSIONS

Mineralized soft-tissue structures preserved in the skin envelope, terminal ungual phalanx sheath and tendon resemble those observed in extant sections of analogous avian skin and tendon (Abdalla 1979; Weir & Lunam 2004; Adams & Organ 2005). The skin of extant vertebrates is commonly composed of two different tissues that are closely apposed to each other: a surface epidermis (constructed from multiple layers of epithelial cells) and an underlying dermis composed of dense connective tissue. Skin-like structures observed in MRF-03 suggest that an epidermis (constructed from multiple layers of epithelial cells) and an underlying dermis composed of dense connective tissue. Skin-like structures observed in MRF-03 suggest that the epidermis has been partially preserved in the area sectioned. Cell-like structures observed via BSEI and microprobe imaging (figures 2c and 3, respectively) support the potential presence of an epidermal layer comparable to extant vertebrates. The size of the cell-like structures (approx. 5–30 μm) is within the size range expected for skin cells (Fusenig 1986; Litzgus et al. 2004). The imaged partial epidermal
Figure 7. Stingray FTIR (reflection mode) images (left) and spectra (right) of a sample taken from a section of ossified epaxial tendon from MRF-03. This shows a cross section through three Haversian canals. (a) Map of the intensity of adsorption at 1170 cm$^{-1}$—crosshairs on the map (left) show from where the spectrum (right) has been selected. The Haversian canals within the apatite are clear. (b) An approximately 20 µm thick carbon dioxide-rich layer on the apatite is evident, again crosshairs on the map show where the spectrum (right) originates from. (c) Absorption bands at approximately 1770 cm$^{-1}$ noted at several places on the sample mapping them showed clear structural control and suggests that organic material may persist associated with the canals.
thickness (3.5 mm) sets a minimum constraint for this organism and is comparable to that of several vertebrates such as humans (0.8–1.5 mm) (Matthews 1986), rhinoceros (15–25 mm) (Shadwick et al. 1992), hippopotamus (15–20 mm) (Shadwick et al. 1992) and elephant (10–15 mm) (Harkness & Harkness 1965).

Chemical mapping and amino acid analyses of MRF-03 clearly indicate that the composition of the preserved specimen differs from that of the surrounding sediment and maintains structures strongly reminiscent of soft tissue. Rapid burial, combined with methanogenesis in a depleted oxygen environment, contributed significant bicarbonate to the system. Intensely reducing porewaters, generated by the decay of plant material, caused oxidized iron species to be reduced and feldspar and rock fragments to partially dissolve. The reduced iron in solution rapidly replaced the soft tissue with carbonate minerals with dissolved silica lining the Havensian canals of the tendon. Mineralization apparently outpaced microbial decay processes, thus ensuring high-fidelity preservation of some integument structures. This rapid mineralization also ensured that some breakdown products of organic molecules at the point of burial, whether endogenous to MRF-03 or of microbial origin, were preserved within the mineral matrix.

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P.L.M. did FTIR analysis and interpretation; A.M. provided MALDI analytical assistance; E.J. carried out size separation, PAGE and MALDI-MS analyses; M.B. and O.R. carried out further MALDI-MS and LC-ESI-MS analyses; J.H.S.M. did optical and BSEI imaging; J.M. completed CL imaging and isotopic analysis; T.L. found the specimen and contributed material; P.L.M. performed ESEM and EMP and assisted B.E.v.D. with Py-GCMS analyses, managed the analytical programme and wrote the manuscript; R.A.W. planned the EMA, FTIR and XRD analyses, analysed the data and co-wrote the manuscript. All authors discussed and commented on the manuscript.

The authors declare no competing financial interests.

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