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First histochemical examination of a Miocene ostrich eggshell with the oldest mineral-bound peptides

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Abstract Because ancient proteins have a higher preservation potential than ancient DNA, proteomic studies can help shed light on the biology of some extinct biological groups that are beyond the reach of the field of ancient DNA. The oldest peptide discovered so far is part of the protein struthiocalcin (SCA-1) involved in eggshell mineralization and found within an ostrich egg from the Late Miocene Linxia Basin of Northwest China. It was originally hypothesized that SCA-1 was evenly distributed within the eggshell and was able to enter the fossil record for so long, because it was bound to calcite crystals. We conducted histological, scanning electron microscopy and Raman spectroscopic analyses on this same fossil egg to test if any protein or organic matter could be observed within specific regions of the eggshell and indeed bound to calcite crystals. Our results show that the eggshell is made entirely of calcite except at the base layer, which is made of mammillary knobs at least partially made of apatite. These knobs were secondarily phosphatized during diagenesis. After decalcification of this material, the fossilized mammillary knobs showed fibrous residues consistent in location and morphology with remnants of original organic material forming a network. This network was similar to the organic matrix observed in an extant ostrich eggshell with this same method. The results here suggest that SCA-1 may have been concentrated at the mammillary knobs, rather than evenly throughout the eggshell. Phosphatization may be another taphonomic process that favors organic preservation in deep-time. The paleoclimate and taphonomic environment of the Linxia Basin may have provided favorable conditions for the molecular preservation of this egg. More in-depth histochemical and mineralogical analyses will certainly increase our understanding of organic and ancient protein preservation in this basin.

Key words fossil organics, struthiocalcin, apatite, phosphatization, ostrich eggshell, ancient proteins

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1 Introduction

The analysis of ancient proteins is an emerging field in paleontology and paleoanthropology that can reveal insights into the physiological, ecological and evolutionary traits of extinct organisms, including our own ancestors (Hendy et al., 2018; Thomas and Taylor, 2019; Warren, 2019; Hendy, 2021). Among ancient proteins, proteins involved in biomineralization specifically show a good preservation potential (Demarchi et al., 2016). Recently, Demarchi et al. (2022) extracted a polypeptide from a fossilized ostrich eggshell found in the Linxia Basin in Northwest China through Liquid Chromatography with tandem mass spectrometry (LC-MS/MS), representing the oldest ancient peptide sequence found to this day, dating back to 6–9 Ma (Demarchi et al., 2022). This polypeptide found by Demarchi et al. (2022) is part of the protein called struthiocalcin SCA-1, homolog to ovocleidin OV-17 of the chick (Arias et al., 2007; Gautron and Nys, 2007; Gautron et al., 2021). Molecular dynamics analysis shows that SCA-1 is tightly bound to calcite particles, making the polypeptide more stable and resistant to degradation (Demarchi et al., 2016). The authors suggested that the binding to calcite crystals is one of the reasons why the protein survived in deep time. They also suggested that the protein was distributed evenly among the layers of the eggshell (Demarchi et al., 2016, 2022).

Nowadays, there are many hypotheses about how molecules (e.g., DNA, proteins) are preserved in deep-time within tissues. Besides what was proposed for SCA-1, it is thought that dry and cold environments would slow down the degradation of organic matter and were considered to be beneficial to the preservation of ancient proteins (Kendall et al., 2018). It is believed that dehydration favors the preservation of non-mineral-bound polypeptide chains (Collins and Riley, 2000). However, without histological analyses, these hypotheses will remain hypotheses. A unification of the fields studying and sequencing ancient biomolecules and paleohistology is necessary to answer all of these questions. Here, we start to test pre-existing hypotheses proposed by molecular paleontologists and experts in ancient molecules in this eggshell bearing the oldest peptide known to date. To test the calcite-bound peptide hypothesis, we decided to analyze the histology (with ground sections and Scanning Electron Microscopy, SEM), and the chemistry (with Energy-dispersive X-ray spectroscopy, EDS) of this same egg in an adjacent eggshell fragment. Additionally, we removed all of the calcite in another fragment using the decalcifying solution EDTA (ethylenediaminetetraacetic acid) to visualize potential organic matter left when all of the calcite was dissolved. Overall, the main goal of this study was to improve the understanding of peptide preservation in this eggshell and its mode of fossilization.

2 Material and methods

2.1 Material

Two fragments were sampled from the fossil ostrich eggshell (*Struthio linxiaensis*, IVPP V26107) from the Late Miocene Liushu Formation of the Linxia Basin of Gansu Province in Northwest China (Demarchi et al., 2022). One fragment was used for petrographic ground-sectioning and SEM-EDS analyses and another one was used for making demineralized paraffin section and histological stains. Extant common ostrich (*Struthio camelus*) eggshell fragments were collected from the IVPP collections for both ground and demineralized paraffin sections and used for comparison with the fossil material. Because of the contrasting material properties, the soft egg membrane of the extant eggshell was removed in the fragment used for ground-sectioning.

2.2 Petrographic ground-sectioning

The fragments of both fossil and extant eggshell were embedded in EXAKT Technovit 7200 (Norderstedt, Germany) one-component resin, which was cured for 12 hours. The embedded blocks were then mounted onto glass slides. The sections were cut with an EXAKT 300CP accurate circular saw, and ground and polished with the EXAKT 400CS grinding system (Norderstedt, Germany) until the desired optical contrast was reached around 30–50 μm thickness. The sections were observed under natural and polarized light with a Nikon eclipse LV100NPOL microscope and photographed with a DS-Fi3 camera and the build-in NIS-Element v4.60 software.

2.3 Demineralized paraffin sections and histological staining

The fragments were embedded in 3% agar (AoBoXing Product 01-023), and demineralized with EDTA (Invitrogen, Thermo Fisher Scientific, 0.5 M, pH 8.0) for 3 days, until the eggshell fragment became transparent. Then the agar blocks were washed with water to remove any left-over EDTA and subsequently subjected to routine paraffin section protocol (Schweitzer et al., 2016; Bailleul et al., 2020), including dehydration, clearing in xylene, and paraffin infiltration and embedding. Sections were cut at 5 μm on a rotary microtome (Leica Biosystems RM2265), placed into a warm water bath (at about 44 °C) added with water bath adhesive (Electron Microscopy Sciences Cat.#71303-01) and mounted on charged slides (Superfrost Plus, Fisher Scientific). The slides for the SEM and EDS examinations were simply deparaffinized in different solutions of xylene for about 15 min. Some other slices were stained with the standard alcian blue stain for the organic matrix of the eggshell following the protocol used in Schweitzer et al. (2016). Stained sections were observed under transmitted and polarized light using the same equipment and software as the ground-section slices.

2.4 SEM and EDS

SEM images and Energy-dispersive X-ray spectroscopy (EDS) of the ground section slices and the deparaffinized demineralized paraffin section slices of both fossil and extant samples were taken at the Chinese Academy of Geological Sciences (Beijing) using FEI Quanta 450 (FEG) at 20 kV without coasting. BSE (back-scattered electrons) modes were applied to the slices.

2.5 Raman spectra analysis

Spot Raman analyses were performed by a Alpha300R Raman spectrometer with a 600 grooves/mm grating and a CCD detector. Data were collected using a laser wavelength of 532 nm and a laser power of 15 mW. The spectra were obtained in the range from 100 to 3900 cm^{-1} at an exposure time of 0.4 s and two data accumulations on the eggshell cross-sections. Acquisition time was 0.4 s for one spot.

3 Results

3.1 Paleohistology, SEM and EDS on the ground sections

The fossil eggshell IVPP V26107 has a thickness of 3.15 mm, which is significantly greater than that of the extant common ostrich eggshell (2.02 mm). The ground section of the fossil eggshell under transmitted light reveals at least two layers from the inside out: a

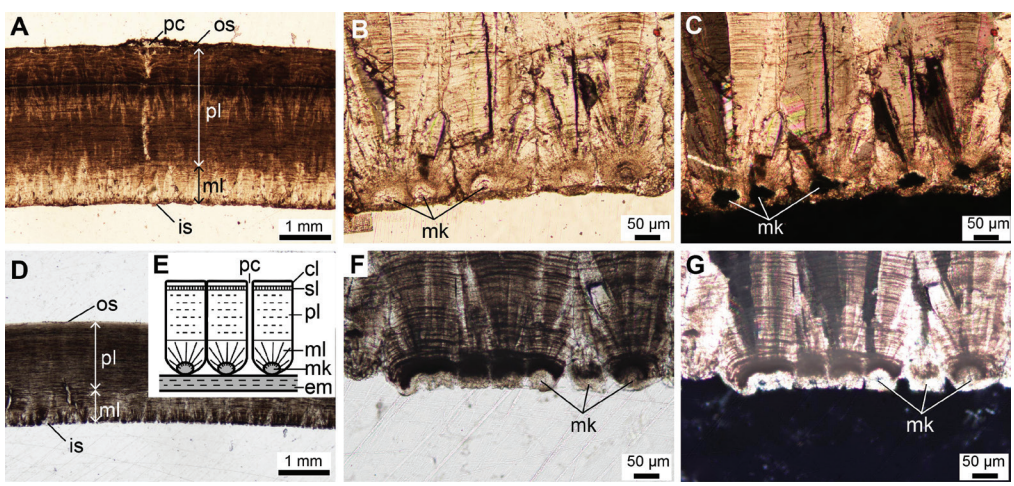

Fig. 1 Histology of the fossil and the extant ostrich eggshells

A. image of a ground section of the fossil eggshell IVPP V26107; B, C. close-up within the mammillary layer of V26107 (B) and its corresponding polarized light image (C) showing the optical extinction of the mammillary knobs; D. image of the ground section of an extant common ostrich eggshell; E. illustration of the structure of avian eggshells; F, G. close-up of the mammillary layer in D (F) and its corresponding polarized light image (G)

Abbreviations: cl. cuticle layer; em. egg membrane; is. internal surface; mk. mammillary knob; ml. mammillary layer; os. outer surface; pc. pore canal; pl. palisade layer, sl. surface crystal layer

mammillary layer and a palisade layer (Fig. 1A). A pore connects the inner and outer surfaces of the shell (Fig. 1A). The lower quarter of the shell (the mammillary layer) is lighter in color than the upper part, which has a thin dark layer on the inner surface (Fig. 1A). This thin dark layer corresponds to the location of the mammillary knobs (Fig. 1B). Under polarized light, the center of the knobs shows an extinction pattern similar to the resin used to embed the samples

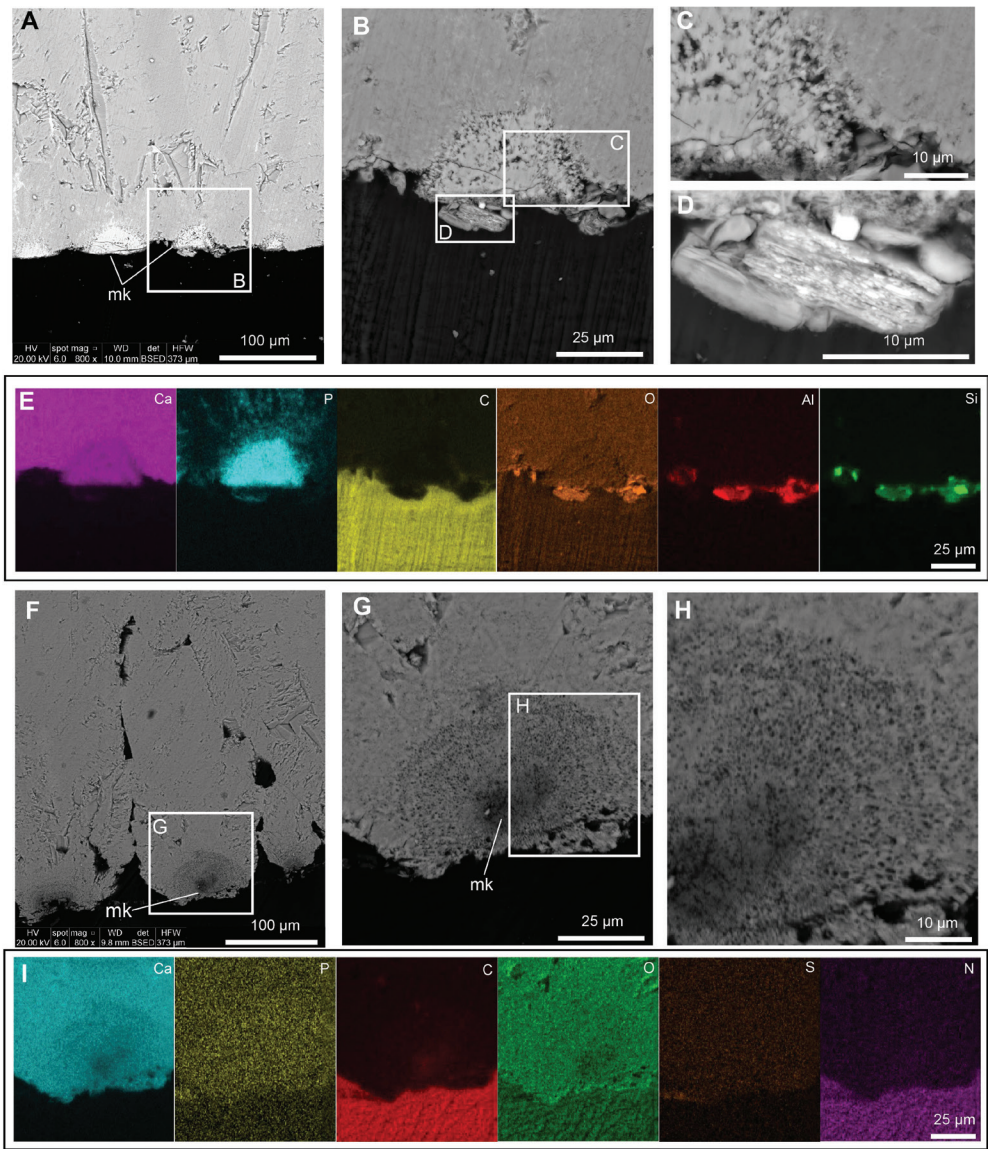

Fig. 2 SEM and EDS of the mammillary knobs of the fossil and extant ostrich eggshell ground-section slices
A–E. backscattered electron image (A), close-up of the mammillary knob (B–D),
and EDS analysis (E) on B of ground-section slice of IVPP V26107, showing a high concentration of phosphorus within the mammillary knob; F–I. backscattered electron image (F), close-up of the mammillary knob (G–H), and EDS analysis (I) on G of a ground-section slice of the extant ostrich eggshell
mk. mammillary knob

and quite distinct from the calcite crystals of the eggshell (Fig. 1C). This is also observed in the extant ostrich eggshell (Fig. 1G).

The back scattered electron (BSE) images of the ground section show that both the fossil and extant eggshells are dense, except for the mammillary knobs which are relatively porous (Fig. 2A–D, F–G). EDS analysis revealed calcium-rich elements throughout both the fossil and extant eggshells (Fig. 2E, I), consistent with calcite as its major component (Hincke et al., 2012). In the fossil eggshell IVPP V26107, the pores of the mammillary knob are less in number but bigger in size than that of the extant ostrich eggshell (Fig. 2C, G). The mammillary knobs of V26107 are made of calcium (Ca), some oxygen (O), and are highly enriched in phosphorus (P) (Fig. 2E). Raman spectra analysis on the mammillary layer of V26107 showed the presence of both calcite and apatite in the knobs, while only calcite was found in other parts of the mammillary layer.

In the extant ostrich eggshell, the core of the mammillary knob shows higher C content and lower Ca content than other parts, and a relative low density indicated by the darker color (Fig. 2F–H). Although there are some P in the extant specimen, the concentration is relatively low, and the distribution is even without local enrichment (Fig. 2I) unlike what is seen in the fossil. Contrary to IVPP V26107, Raman analysis did not find any apatite in the extant ostrich eggshell, either in the mammillary layer nor in the palisade layer, which is consistent which results of previous studies (Cusack et al., 2003; Yang et al., 2018).

Additionally, some layered material adheres to the mammillary knob of the fossil eggshell (Fig. 2B–D), which is rich in O, aluminum (Al) and silicon (Si) elements (Fig. 2E), indicating that it may be clay and silica that constitute sediments, consistent with the burial environment of the Liushu Formation (Deng et al., 2004b). At the bottom of the EDS images of both the fossil and the extant ostrich eggshell, the resin used for embedding shows significant high signal of carbon (C), indicated as bright yellow in the fossil sample (Fig. 2E) and bright red in the extant sample (Fig. 2I).

3.2 Paleohistology, SEM and EDS on demineralized paraffin sections

The second fragments of both fossil and extant eggshells were decalcified in EDTA then transformed into 5 μm sections, deparaffined with xylene and then stained with alcian blue (Fig. 3). After decalcification most mineral matrix of both the fossil and extant eggshell dissolved, including calcite and apatite. Some residues that have a radial and fan-shaped appearance can be seen at the internal surface of the fossil eggshell (Fig. 3A, D). These residues are consistent in morphology, distribution and size of the mammillary knobs of both the fossil and extant ostrich eggshell (Figs. 1B, F; 3B), while all the other layers of the eggshell were completely dissolved in the EDTA solution or did not adhere to the slide (Fig. 3A). The micro-fibers that are found in these residues seem connected to each other forming a network (Fig. 3D–E). In the demineralized section of the extant ostrich eggshell, the knobs are connected to the soft egg

membrane with a more regular shape (Fig. 3B, C). The core of the knobs of the fossil sample seems to be empty after demineralization (Fig. 3A, C, D) and was probably also dissolved in the EDTA solution, just as the other layers of the eggshell were.

Under SEM, residual calcite crystals (rich in calcium) in the middle of fragment of V26107 is indicated by the bright yellow in Fig. 4. We observed a fibrous matter that was attached to the residual calcite crystals (indicated by the white arrows, Fig. 4). The fibrous matter is approximately 42 microns long and 6.5 microns wide at its widest point, tapering at both ends and slightly wider in the middle, where it attaches to a calcite crystal (Fig. 4A–B). The element composition of this fiber is significantly different from that of the surrounding calcite, with very few calcium but rich in carbon, suggesting that it may be a remnant of original organic material that was slightly calcified.

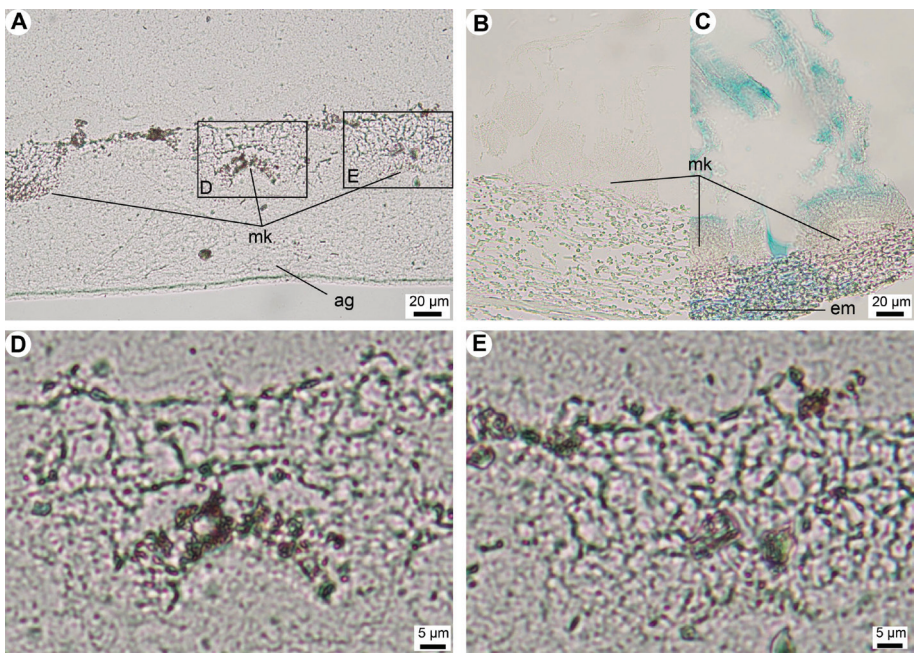

Fig. 3 Demineralized paraffin section of the fossil (A, D and E) and extant ostrich eggshells (B and C)
Image of a paraffin section of fossil eggshell IVPP V26107 left completely unstained (A) and extant ostrich eggshell (B) stained with alcian blue (C). Close-ups of the fossil residues show a fibrous network most likely representing ancient proteins or at least, some fossilized organic matter (D–E)
Abbreviations: ag. agar; em. egg membrane; mk. mammillary knob. B and C share the same scale bar

3.3 Raman spectra analysis on the ground sections

Raman spectra of the mammillary knob on the ground section of both fossil and extant ostrich eggshell show obvious band at 283 cm^{-1} and 1085 cm^{-1}, which correspond to the calcite vibrational pattern, and consistent with calcite, the main mineral in eggshells (Fig. 5). Raman spectra of the fossil samples also show a clear band at ~961 cm^{-1} (Fig. 5), which can be attributed to the symmetric stretching vibration of the P-O bond in phosphate radical (PO_4^{3-}). This

observation, along with the results from the SEM and EDX analysis (Fig. 2), indicates that the mammillary knob of the fossil eggshell contains not only calcite, but also apatite.

The band at 1434 cm^{-1} and 2933 cm^{-1} in both fossil and extant eggshell samples may represent some organic matter, which is consistent with the band in the resin used for sample embedding (Fig. 3), rather than indicating any endogenous organic matter. The resin may penetrate the sample during embedding due to the porous feature of the mammillary knob observed by SEM (Fig. 2). Considering the definitive evidence for the presence of endogenous organic matter in this fossil and extant specimen (Figs. 1, 3, 4) (Demarchi et al., 2022), this implies that the Raman spectroscopy may not be effective in detecting the presence of organic matter in such samples embedded in resin, or the endogenous organic matter (the protein or peptide) may be too traced, to be identified by Raman spectroscopy. It also reminds us that we need to be careful when testing the present of organic matter in this kind of samples.

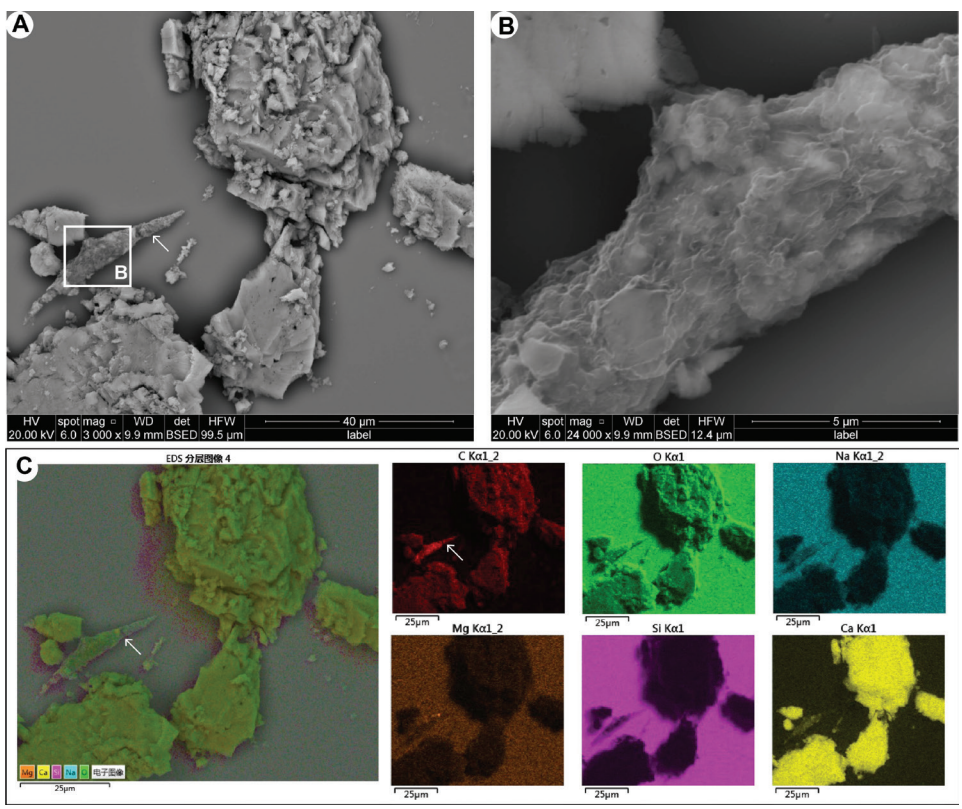

Fig. 4 SEM and EDS of demineralized paraffin section slice of IVPP V26107
Backscattered electron image of demineralized products on a paraffin section slice of IVPP V26107 (A), its close-up (B) and EDS analysis (C) on the same image, showing the fibrous matter rich in carbon and slightly calcified (indicated by the white arrow), attached to crystal of calcite

4 Discussion

4.1 Phosphatization of the mammillary knobs of the fossil eggshell

The results of EDS and Raman spectroscopy analyses show that there is apatite concentration specifically at the mammillary knobs of IVPP V26107 (not in the palisade layer nor anywhere else in the eggshell) (Figs. 2, 3, 5). Since no apatite has ever been reported in the mammillary and palisade layers of extant avian eggshells (which is also supported by the Raman data of this study, showing no apatite anywhere in the fragment of the extant ostrich egg, Fig. 5), and apatite is only found in the cuticle layer of extant avian eggshells (Cusack et al., 2003; Yang et al., 2018), the apatite in V26107 is diagenetic. The small amount of phosphorus found here in the extant ostrich eggshell comes from the organic matrix of the eggshell.

Phosphatization is a relatively common phenomenon during the fossilization of the shell of soft eggs probably through apatite replacement of the membrana testacea (shell membrane). This was observed in the egg of *Hamipterus* from the Early Cretaceous (Li et al., 2022), the egg of *Lufengosaurus* from the Early Jurassic (Stein et al., 2019), and in *Antarcticoolithus* from the Late Cretaceous (Legendre et al., 2020). In extant avian eggshells, the mammillary knobs have a significantly higher organic matter content than other parts of the eggshell (Nys et al., 2004; Solomon, 2010; Hincke et al., 2012). Therefore, the mechanism of phosphatization involved in the egg of *Struthio linxiaensis* studied here may have been similar to soft egg fossilization, where apatite grew onto the template formed by the original organic matter at the molecular scale (Zhang et al., 2011; Li et al., 2022).

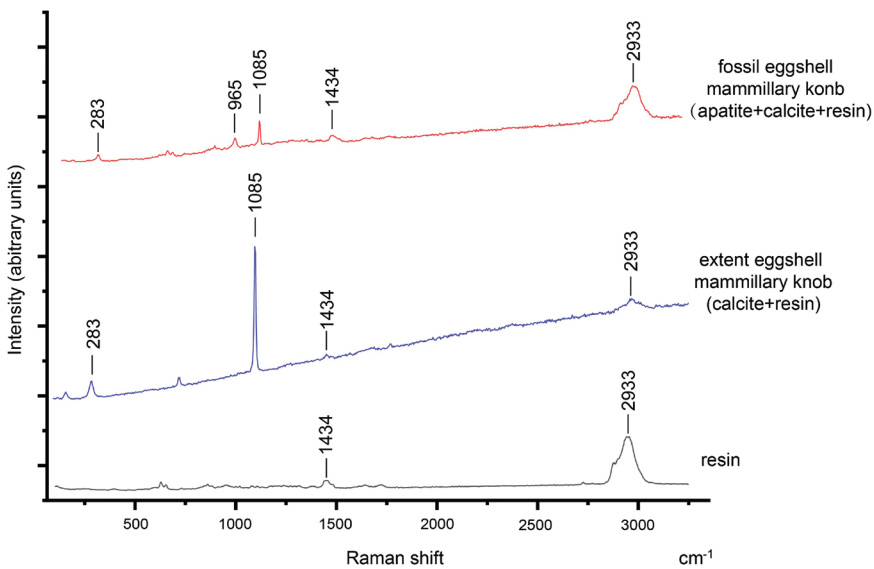

Fig. 5 Raman spectra point analysis results of the mammillary knob of fossil and extant ostrich eggshell
Showing the concentration of apatite in the mammillary knob of IVPP V26107

4.2 Preliminary insights on organic preservation

In this study, we found some fibrous residues after decalcification from the fossil ostrich eggshell similar in morphology, location, and size with the mammillary knobs of the extant eggshell (Figs. 3, 4). Since the decalcification solution removed calcium in minerals like calcite and apatite, and since the EDS analysis excluded the presence of silica (Fig. 2), these residues are most likely organic, probably remnants of the original proteinaceous fibrous network found within the mammillary knobs.

It was suggested in a previous study that SCA-1 protein was evenly distributed among the layers and bound to calcite crystals (Demarchi et al., 2016). However, our results suggest a different story.

Among the mineralized eggshell in extant birds, the mammillary knob is the site of initiation of calcification and is rich in organic matter (Nys et al., 2004; Solomon, 2010; Hincke et al., 2012). SCA-1 and its homologous protein OV-17 are not distributed evenly throughout the shell matrix but concentrated in the mammillary knobs (Hincke et al., 1995; Gautron et al., 2021). The residue that we have found here in the mammillary knobs of the demineralized fossil ostrich eggshell (Fig. 3), is consistent with the abundance of organic matrix of the mammillary knobs and the concentration of SCA-1 in extant avian eggs. Hence, the ancient peptide belonging to SCA-1 that was found by Demarchi et al. (2022) in the very same egg that we re-analyzed here may not be evenly distributed among the layers. Instead, it may be concentrated within the mammillary knobs.

Additionally, since the mammillary knobs of V26107 were enriched in phosphorus (Fig. 2E) and contained apatite (Fig. 5), all our results and knowledge from the literature combined together suggest that phosphatization may also have contributed to the survival of the polypeptide found in Demarchi et al. (2022).

Phosphatization, as an important taphonomic process of fossil preservation, is the finest taphonomic mode that can preserve subcellular structures (Briggs et al., 1993; Schiffbauer et al., 2014). Some organic molecules such as proteins can interact with apatite crystals and become embedded or adsorbed within the mineral structure, similar to what happens during the formation of bones and teeth (e.g., Iline-Vul et al., 2020; Hong et al., 2022). It has been shown experimentally that phosphatization of soft tissues can occur within several weeks (Briggs and Kear, 1993). This prevents the decomposition of organic matter early during burial, further facilitating long-term preservation.

4.3 Hypothesized diagenetic pathways of the ostrich eggshell from the Linxia basin

During phosphatization, organic tissues such as the egg membrane are a potential source of phosphorus (Schiffbauer et al., 2014). The phosphorus could also come from the environment or decaying organic matter (Orr, 2014), and the abundant animal bone fossils found in the strata of the Linxia Basin (Bergmann et al., 2010). As a closed structure,

the eggshell can isolate external oxygen and microorganisms, which is conducive to the preservation of organic matter (McCoy, 2014). During the Late Miocene period, the uplift of the Tibetan Plateau caused monsoon changes, resulting in arid climates in the central regions of Asia, including the Linxia Basin (Li and Fang, 1999; Deng, 2004a, 2009; Liu and Dong, 2013). A dry environment is also thought to be a factor favoring the preservation of ancient proteins, since the breakdown of polypeptide chains into amino acids requires the participation of water (Demarchi et al., 2016).

Combining the microstructural results with EDS and Raman analyses, our study may propose a diagenetic pathway for the phosphatization of the original organic matter in this ostrich eggshell, as well as other organic preservation in Linxia Basin (Fig. 6) (Li et al., 2021). The process of avian embryonic development leads to the resorption of the mammillary knobs and the separation of the hard eggshell from the shell membrane (Hincke et al., 2018). In V 26107, the mammillary knobs are well preserved, indicating that there was no embryo ever developed in this egg, and the eggshell including the shell membrane were held together. The initial decomposition of the organic matter in the eggshell may have been prevented by the arid or semiarid environment of the Late Miocene of Linxia Basin. After the egg was buried by the sediment brought in by seasonal precipitation, phosphatization may have occurred quite rapidly and phosphate minerals may have replaced some of the organic material within the mammillary knobs. This most likely helped preserved peptides within this eggshell, such as SCA-1, specifically found with LC-MS/MS by Demarchi et al. (2022). Further work is needed to verify this hypothesis to better understand modes of organic preservation in this eggshell and in the entire Linxia Basin.

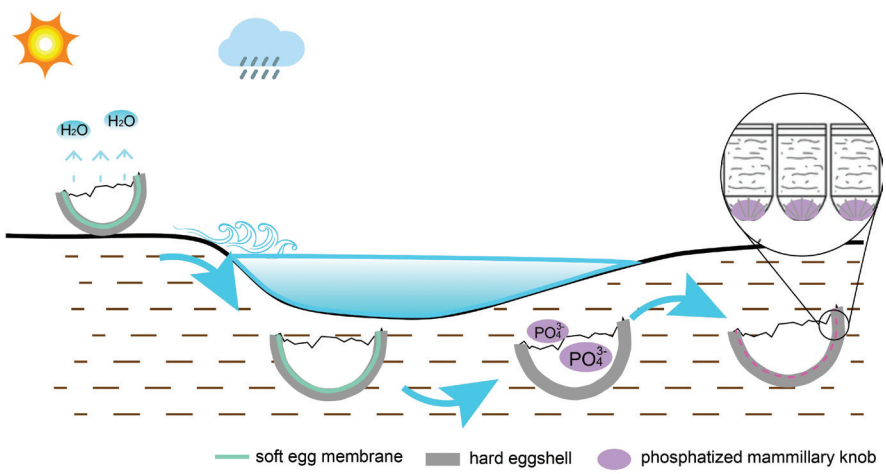

Fig. 6 Hypothesized diagenetic pathways of the ostrich eggshell from Linxia Basin

5 Conclusions

This study reports the preservation and unique phosphatization process of mammillary knobs in a fossil ostrich eggshell from the Linxia Basin, representing a case of avian eggshell phosphatizing during fossilization. We suggest that the protein distribution in this fossil eggshells is concentrated within the mammillary knobs as it is in extant bird eggshells, instead of homogeneously distributed thoughout the eggshell. We propose that phosphatization, perhaps in addition to calcite binding of some proteins, may also play a role in preserving peptides over geological time. This is a preliminary study and additional in-depth analyses involving protein stains and immunohistochemistry will be necessary to confirm our phosphatization and our new apatite-bound peptide hypothesis. We further propose a hypothetical diagenetic pathway for organic preservation in this eggshell and for the entire Linxia Basin. We encourage further analysis of more specimens from the same strata within the Linxia Basin, to provide more evidence of molecular preservation, and to fully understand mechanisms for organic preservation in deep-time.

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保存最古老矿物结合多肽的中新世鸵鸟蛋壳化石首次组织化学研究

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摘要：古蛋白质比古DNA具有更高的保存潜力，因此蛋白质组学研究可以帮助阐明一些超出古DNA研究领域的灭绝生物群体的生物学特征。迄今为止最古老的多肽发现于中国西北地区

晚中新世临夏盆地的鸵鸟蛋壳化石中，是与蛋壳矿化相关的蛋白质struthiocalcin (SCA-1) 的一部分。前人认为SCA-1在蛋壳中均匀分布，并因其与方解石晶体结合的特性而得以在地质历史中长时间保存。本次对同一鸵鸟蛋壳化石进行了组织学、扫描电子显微镜和拉曼光谱分析，发现蛋壳内侧锥体层的晶核含有部分磷灰石，其他部位则完全由方解石构成；这些晶核部分应当是在成岩作用过程中经历了磷酸盐化。在对该化石蛋壳样品脱钙处理后，其锥体层晶核部分存在残留物，呈现网络状纤维结构，其位置和形态与现生鸵鸟蛋壳中脱钙后残留的有机质相似。结果表明，该化石蛋壳中的古多肽可能集中保存在锥体层晶核处，而非在整个蛋壳中均匀分布。磷酸盐化可能是另一个有利于有机物长期保存的埋藏过程。临夏盆地的古气候和埋藏环境可能为该古蛋白分子的保存提供了有利的条件。建议在未来研究中进行更深入的组织化学和矿物学分析，以进一步了解该盆地有机质和古蛋白的保存机制。

关键词：化石有机质，生物矿化蛋白，磷灰石，磷酸盐化，鸵鸟蛋壳，古蛋白

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