Extreme biomineralization: the case of the hypermineralized ear bone of gray whale (*Eschrichtius robustus*)

Marcin Wysokowski¹,² · Iaroslav Petrenko² · Roberta Galli³ · Christian Schimpf⁴ · David Rafaja⁴ · Jana Hubalkova⁵ · Christos G. Aneziris⁶ · Sergey Dyshlovoy⁶,⁷ · Gunhild von Amsberg⁶,⁸ · Heike Meissner⁹ · Yuri M. Yakovlev¹⁰ · Konstantin R. Tabachnick¹¹ · Allison L. Stelling¹² · Hermann Ehrlich²,¹³

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Abstract

Selected hypermineralized bones (rostrum and tympanic bullae) have yet to be characterized for diverse species of whales (Cetacea). Hypermineralization in these structures is an example of extreme biomineralization that, however, occurs at temperatures around 36 °C. In this study we present the results of analytical investigations of the specimen of tympanic bulla isolated from gray whale (*Eschrichtius robustus*) for the first time. Examination of the internal surface of the bone mechanically crushed under a press revealed the presence of a lipid-containing phase, which did not disappear even after complete demineralization of the bone material. Additionally, analytical investigations including CARS, ATR-FTIR, Raman and XRD confirmed the presence of carbonated bioapatite and a collagen-lipid complex as the main components of this up to 2.34 kg/cm³ dense bone. Our experimental results open the way for further research on understanding of the principles of hypermineralization in highly specialized whale bones.

Keywords Biological materials · Biomineralization · Hydroxyapatite · Tympanic bulla · Whale bones

*Marcin Wysokowski  
Marcin.Wysokowski@put.poznan.pl

*Hermann Ehrlich  
Hermann.Ehrlich@esm.tu-freiberg.de

¹ Faculty of Chemical Technology, Institute of Chemical Technology and Engineering, Poznan University of Technology, Poznan, Poland
² Institute of Electronic and Sensor Materials, TU Bergakademie Freiberg, Freiberg, Germany
³ Clinical Sensing and Monitoring, Department of Anesthesiology and Intensive Care Medicine, TU Dresden, Dresden, Germany
⁴ Institute of Materials Science, TU Bergakademie Freiberg, 09599 Freiberg, Germany
⁵ Institute of Ceramic, Glass and Constructions Materials, TU Bergakademie, Freiberg, Germany
⁶ Department of Oncology, Hematology and Bone Marrow Transplantation with Section Pneumology, Hubertus Wald-Tumorzentrum, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
⁷ Laboratory of Pharmacology, A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia
⁸ Martini-Klinik, Prostate Cancer Center, University Hospital Hamburg-Eppendorf, Hamburg, Germany
⁹ Department of Prosthetic Dentistry, Faculty of Medicine, University Hospital Carl Gustav Carus of Technische Universität Dresden, Fetscherstraße 74, 01307 Dresden, Germany
¹⁰ A.V. Zhirmunsky National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russian Federation
¹¹ P.P. Shirshov Institute of Oceanology of Academy of Sciences of Russia, Moscow, Russian Federation
¹² Department of Biochemistry, Duke University Medical Center, Durham, NC, USA
¹³ Center for Advanced Technology, Adam Mickiewicz University, Poznan, Poland
1 Introduction

Biomineralization, demineralization, and remineralization are the fundamental scientific directions within such interdisciplinary research field as modern biomineralogy. Biomineralization as a biogeochemical phenomenon has been naturally occurring since life began. From a scientific point of view, close attention was traditionally paid to such areas as mechanisms and principles of biomineralization, problems of the vital effect, biomineralizing phyla with respect to evolutionary history and the Cambrian explosion, and, more recently, to principles of control and designies—including shaping of crystals with biomolecules, and inspiration for biomimetics and biomaterials science [1–4].

The term “extreme biomineralization” was proposed by us in 2017 for the first time [3]. Currently attractive studies on the origin of life and environmental issues are related to hydrothermal synthesis or hydrothermal chemistry. Surprisingly, extreme biomineralization, which seems to be one of the most ancient phenomenon that naturally occurred under harsh environmental conditions, is less considered. Extreme environments by definition are characterized by “conditions that are far outside the boundaries in which most organisms live comfortably. Conditions include: pH, air pressure, temperature, salinity, radiation, dryness (desiccation), and oxygen levels” [5]. Nowadays, extreme biomineralization is discovering how the structures and functions of organisms survive under natural albeit extreme conditions, and have inspired new forms of artificial biomineralization and technology for extreme biomimetics [6–14].

However, one of the special scientific niches within extreme biomineralization deals not with extreme environments, but with such phenomenon as hyperbiomineralization that occurs under ambient conditions, for example at 36.6 °C in whales. It includes both pathological and non-pathological formation of hypermineralized mineral phases within such biocomposite-based constructs as bones and teeth. Our attention is focused on selected bones of whales (Cetaceans). One of the open questions till now is how the manipulation of inorganic phases by organic macromolecules enables cetaceans to create enormous biominerals, where occlusion of biomacromolecules within individual crystals generates superior hyperdense and hierarchically structured architectures with specific mechanical properties which definitively played significant roles in the survival of the largest mammals on Earth over millions years of their evolution. For example, the rostral part of the composite calvarium bones of an adult male of the ziphiid whale species, Mesoplodon densirostris, yielded among the highest values for density (BMD) ranging from 3.245 to 4.481 g/cm² [15], mineralization (86.7%), and compactness (99%) yet reported [16]. Recent data reported by Li et al. [17] represent carbonated hydroxyapatite as the main inorganic component that contain over 95 wt% mineral. The biomechanical behavior of the rostrum is that of an exceptionally rigid (Young’s modulus up to 46.9 GPa), hard (hardness > 200 VHN) but also very brittle (bending strength > 59 MPa) biomaterial [18]. Far from improving resistance to fracture, densification of the rostrum increases its brittleness, facilitating rapid and extensive propagation of cracks. This poor mechanical behavior is a general characteristic of very compact and/or hypermineralized bones.

Other examples of hypermineralized bones are ear bones (otic bones) of whales including tympanic bulla. For example, the mineral content of this bone in fin whale (Balaenoptera physalus) was measured as 86.4% [19]. In spite of few publications concerning its structure [20], morphology [21–25] including application of the modern microcomputed tomography (µ-CT) [25–27], and histology [28, 29] most scientific attention has been focused on the materials properties [19] as well as functional role of this highly specialized bone in ultrasonic hearing and echolocation of whales [30–36]. The large relative and absolute sizes of the ear bones of the sperm whale embryos (as well as the baleen whales) are striking. For example, in a 73-mm long embryo, the ear bone had a size of 27 mm, and in the prenatal (365 cm long) it already reached the size of the ear bone of an adult animal, which can be explained, apparently, by the same functional load on the hearing organ in newborns and adult whales. It is recognized that “early mineralization of the tympanic bulla allows immediate sound conduction in the aquatic medium and consequently holds potential importance for mother-calf relationship and postnatal survival” [37]. In fin whale material properties of tympanic bulla have been characterized by very low bending strength and impact resistance, however by high modulus (14–31 GNm−2) and high density (2.06 × 10³—2.47 × 10³ kg/m³) [19].

According to the modern hypothesis, “ultra-high matrix mineralization of sperm whale auditory ossicles facilitates high sound pressure and high-frequency underwater hearing” [27]. However, the principles of calcification and the development of such hypermineralized bones as tympanic bulla of whales as well as the corresponding chemical driving forces that pattern the architecture of such unique biominerals remains unknown. In addition, the nature and origin of the “organic matrix” not only with respect to commonly recognized collagenous template need to be studied in details. Obtaining of such data could be crucial for our understanding of remodeling and self-healing of fractured cetaceans tympanic bullae reported in the literature [38]. The mechanism underlying remineralization in vivo is unknown.
We suggest that hyperbiomineralization of ear bones represents an example of forced biomineralization. Forced biomineralization occurs due to objectively developing stereochemical and physicochemical circumstances at the molecular level, when the formation of a crystalline phase on an organic matrix simply becomes inevitable. The challenging task is to elevate the mechanisms leading to such hyperbiomineralization in the body of a whale in parallel with the formation of ordinary or osteoporotic-like bones (i.e. ribs). In this study, we represent the results of analytical investigations on the tympanic bulla of one specimen of north western gray whale (Eschrichtius robustus Lilljeborg, 1861) (Eschrichtiidae: Mysticeti: Cetacea) for the first time. Demineralization was carried for 6 weeks at 37 °C until complete demineralization was reached due to approving with EDX/SEM analysis. Osteosoft® was changed every 24 h to avoid saturation. The organic content was estimated using gravimetric method.

\[
\text{Organic content(\%) } = \frac{m_2}{m_1} \cdot 100\% \quad (1)
\]

whereas, \(m_1\) — mass of sample before demineralization; \(m_2\) — mass of sample after demineralization.

2.3 SDS-PAAG

The demineralized gray whale ear bone sample (Fig. 4b) was cut in into 2×4 mm large fragments and dried for 2 h at RT, followed by the digestion in 100 µL of 0.5 M AcOH buffer containing 0.5% pepsin (w/v) (24 h, 4 °C). The sample was sonicated (5 min, RT) and the protein-containing supernatant was collected using QIAshredder (Cat. No. 79654, QIAGEN) columns and centrifugation (10 min, 10,000 g). The supernatant was collected, lyophilized (2 h, RT) and resuspended in 30 µL of lysis buffer as previously described [45] to generate the sample #2 (see Fig. 10). The control samples of collagen type I (isolated from calf skin, Sigma-Aldrich Cat. No. C9791, sample #3) or pepsin-containing digestion buffer (sample #4) were processed in the same way. The samples were further mixed with loading dye and the proteins were separated using gradient gels as previously described [46]. The gel was washed with water, fixed, stained with colloidal Coomassie G-250 overnight, followed by the destained with water as reported by us before [47].

2.4 ATR-FTIR spectroscopy

Infrared spectroscopy has been used for the qualitative characterization of the bone under study prior and after demineralization of selected specimens. The presence of expected functional group was confirmed by ATR–FTIR (attenuated total reflectance–Fourier transform infrared spectroscopy) and verified using as Nicolet 210c spectrometer (Thermo Scientific, Waltham, USA). The investigation was performed over a wave number range of 1900–500 cm\(^{-1}\) (resolution of 0.5 cm\(^{-1}\)).

2.5 Raman spectroscopy

Raman spectra were recorded using a Raman spectrometer (RamanRxn1, Kaiser Optical Systems Inc., Ann Arbor, USA) coupled to a light microscope (DM2500 P, Leica Microsystems GmbH, Wetzlar, Germany). The excitation of Raman scattering was obtained with a diode laser emitting at a wavelength of 785 nm, propagated to the microscope.
with a 100 µm optical fiber and focused on the samples by means of a 50x/0.75 microscope objective, leading to a focal spot of about 20 µm. The Raman signal was collected in reflection configuration and sent to the f/1.8 holographic imaging spectrograph by using a 62.5 µm core optical fiber. The spectral resolution in the range 150–3250 cm⁻¹ was 4 cm⁻¹. Raman spectra were punctually recorded, using an integration time of 0.5 s and averaging 120 spectra in order to improve the signal-to-noise ratio. Spectroscopic data were analyzed with MATLAB toolboxes (MathWorks Inc., Natick, USA). A baseline procedure was applied in order to remove the background fluorescence signal.

2.6 X-rays diffraction analysis

X-ray diffraction was performed with the purpose of phase identification. The X-ray diffraction pattern was recorded with a Seifert/FPM RD7 diffractometer equipped with a sealed X-ray tube with Cu anode. The experiment was performed in symmetrical Bragg–Brentano diffraction geometry. The powder sample was put on a zero-background holder (Si, < 510> cut). Chips of the isolated organics and collagen standard were fixed, also on a zero-background holder with Vaseline. The diffracted beam passed a set of slits and a graphite monochromator before being detected by a proportional counter. The Rietveld refinement [48] for a more detailed analysis of the sample was conducted employing Maud software package [49].

2.7 X-ray microfocus CT

In order to visualize the three-dimensional microstructure of the whole whale ear bone sample, micro-CT scans were performed using CT-ALPHA (ProCon X-ray GmbH, Sarstedt, Germany) equipped with a 160 kV transmission X-ray tube FXE-160 (Feinfocus GmbH, Harbsen, Germany) and a panel detector Dexela 1512 NDT (PerkinElmer Inc., Santa Clara, USA). The CT device was operated at 150 kV and 75 µA in microfocus mode and a target power of 4.5 W. The exposure time was set to 1.6 s. During a CT-scan 800 radiographs were acquired within 360°. The volume reconstruction was done with the Software Volex 6.0 (Fraunhofer EZRT, Fürth, Germany). The resulting voxel size after reconstruction was 66 µm. The CT scan was visualized using the software VG Studio MAX 2.2 (Volume Graphics GmbH, Heidelberg, Germany).

2.8 Microscopy

The surface morphology and microstructure of the samples were analyzed with advanced imaging and measurement system consisting of Keyence VHX-6000 digital optical microscope and the swing-head zoom lenses VHZ20R (magnification up to 200 x) and VH-Z100UR (magnification up to 1000 x) (Keyence, Osaka, Japan) as well as using Scanning Electron Microscope (XL 30 ESEM, Philips). Samples were covered with a gold layer for 40 s using an Cressington sputtercoater 108 auto. (Crawley, GB).

2.9 Label-free multiphoton microscopy

The multiphoton system was composed by a special laser excitation and a scanning microscope. Two fiber lasers emitting picosecond pulses at 780 and 1005 nm (Femto Fiber Pro NIR and TNIR, Toptica AG, Gräfelfing, Germany) were used to excite the CARS signal of CH2 symmetric stretching vibration (Raman shift = 2850 cm⁻¹), as well as TPEF and SHG signal. A laser scanning microscope (AxioExaminer with LSM7, Carl Zeiss Microscopy GmbH, Jena, Germany) was used to raster the laser beams, which were focalized on the samples by a ×20 water immersion objective with numerical aperture = 1.0, leading to a focal spot of about 0.5 µm (full width half maximum). The CARS signal was collected in the range of 633 to 647 nm, TPEF was always collected in the range 500 to 550 nm and SHG in the range of 381 to 399 nm. All three signal were collected in reflection configuration and used to build intensity images that were afterward merged and are displayed as RGB images (red channel: CARS, green channel: TPEF, blue channel: SHG).

3 Results and discussion

The overview of the tympanic bulla specimen investigated in this study is represented in Fig. 1. The size and shape of this bimemoral-based construct is typical for other tympanic bullae of cetaceans origin reported in the literature previously (see for overview [22, 23]. The images obtained confirm the uneven distribution of the mineral phase and the presence of a difference in the thickness of this bone in different places, which undoubtedly complicates the conduct of comparable biomechanical studies. It is worth nothing that investigations concerning detailed material properties have not been in focus of this work. It is recognized that tympanic in cetaceans are remarkable because of their mineral content, high density and extreme compactness [28]. We have estimated the density of the tympanic bulla of this gray whale as 2.34 g/cm³. These data are in good accordance to that published for cetacean’s tympanic bullae from other species [28, 37]. The content of the organic measured after demineralization of selected tympanic bulla fragments (see, as example Fig. 4b) reach 11.2%.

The specimen of tympanic bulla of the gray whale origin that has been studied here possesses typical, naturally occurring dark yellowish pigmentation. Commonly such
specimens represented in other studies remains to be white in color (see [23]) due to harsh initial chemical treatment using diverse bleaching reagents, or hydrogen peroxide. We have found photographs of similarly pigmented tympanic bullae only in the monograph by Huggenberger and co-workers [26] and in the paper by Yamato and co-workers [38]. To our great surprise, we managed to find this pigmentation after a bone fracture under a mechanical press (Fig. 2 a, b) being located deep inside it (Fig. 2 c).

The investigated bone consists of macroporous material and poses porosity of 6 vol% by the average size of pores 99–70 μm (Fig. 3). Due to relatively low macroporosity this hypermineralized bone can be loaded up to 170 kg in the orientation shown on the Fig. 2 a, exceeding this value will lead to bone destruction.

Large amounts of a lipid-rich phase within channels (Fig. 3b) become well visible on the cleaved bone (Fig. 2 c). It has been suggested previously [19] that in the cetacean’s hypermineralized tympanic bulla the mineral phase is almost entirely coalesced, consequently it was surprisingly to observe the presence of fats within this biological hard tissue construct. Furthermore, also after up to 3 month-long gentle decalcification using Osteosoft-Reagent at 37 °C (Fig. 4) the mineral-free matrix remained pigmented (Fig. 4b). In contrast to other researchers who carried out decalcification of tympanic bulla slices for 1 week using formic acid [29] we have used EDTA-based reagent. This kind of bone treatment ultimately made it possible to obtain an organic framework containing cells as well as a variety of biomacromolecules, including phospholipids, carotenoids and collagen, which have been identified using Raman (Fig. 5), CARS (Fig. 6) and infrared (Fig. 9) spectroscopy. The occurrence of carotenoids within the mineralized bone matrix is unknown, however their origin can be explained. The north western gray whales diet [50] is mostly composed of crustaceans, especially mysids (Mysidacea) [51] which are rich in carotenoids, lipo-glyco-carotenoproteins [52] highly-unsaturated fatty acids and phospholipids [53, 54].

Raman spectroscopy was performed on selected gray whale tympanic bulla fragments prior and after demineralization. The Raman band assignment follows Movasaghi et al. 2007 [55]. Raman spectroscopy performed on the fracture surface of the non-demineralized bulla fragment (Fig. 5a) is dominated by the phosphate bands of HAP at 590 and 960 cm⁻¹, and by the carbonate bands of calcium carbonate apatite at 1073 cm⁻¹ (all indicated by an asterisk in

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**Fig. 1** Reconstructed tomographic 2D-slices (a—c) and volume rendering (d) of the gray whale ear bone under study. The dorsal view clearly shows the bone architecture including characteristic tympanic cavity (d)
Moreover, weak bands typical of organic material are also presented (visible in the magnified spectrum in Fig. 5a). These bands are of proteins and are compatible with collagen. After demineralization, the Raman spectra (Fig. 5b–d) clearly indicate collagen as main component of the protein matrix. For instance, the bands at 815, 856, 876, 920, 940, 1003, 1031, 1245, 1270, 1320, 1342, 1450 and 1655 cm\(^{-1}\) are all assigned to collagen; the bands at 570 and 760 cm\(^{-1}\), as well as in the CH\(_3\) stretching bands at 1885 and 2940 cm\(^{-1}\) also indicate a biochemistry dominated by protein.

However, there are slight differences among regions. While spectra on Fig. 5c and d were acquired in white or only slight yellowish regions (compare with Fig. 2c) and show only bands of collagen, the spectrum acquired from a yellow colored area shown in Fig. 5b also displays bands at 780, 980 and 1077 cm\(^{-1}\), which might be attributed to phosphate groups in phospholipids. A shoulder at about 2850 cm\(^{-1}\) (which is assigned to the CH\(_2\) stretching vibration and is indicated by an arrowhead in Fig. 5b) also suggests increased lipid content in this area. Furthermore, the yellow region is highly fluorescent, which justifies the higher noise in the spectrum. The weak Raman band at 1527 cm\(^{-1}\) may indicate presence of carotenoids, contributing to the yellow coloration.

The same demineralized sample of organic scaffold was investigated by label-free multiphoton microscopy, combining coherent anti-Stokes Raman scattering (CARS),
two-photon fluorescence (TPEF) and second harmonic
generation (SHG). Which tissue structures are visual-
ized by this technique was already investigated in detail
elsewhere [56]. CARS was acquired by tuning the system
to excite the Raman band of CH₂ stretching vibration at
2850 cm⁻¹. Therefore, this signal shows lipids and proteins.
No defined lipid-rich structure were visualized; the CARS
signal appears rather evenly distributed in the extracellular
matrix. SHG displays the fibers of collagen type I with high
specificity. TPEF appears concentrated in the cytoplasm of
cells which may be identified as osteocytes and are scattered
within the protein matrix, being more numerous in the yel-
low regions compared to the white regions (compare Fig. 6a,
c). The yellow regions display a matrix that is formed by col-
lagen as well as a different material, which is characterized
by both CARS and TPEF signal (but no SHG). This may be
related to the different biochemistry also revealed by Raman
spectroscopy. However, the technique does not allow better
identification of this material. The pores already observed
with light microscopy were also visualized by multiphoton
microscopy (indicated by arrowheads in Fig. 6). The struc-
ture composed of concentric layers (see Fig. 6a) appears
similar to that of osteons.

In the powder sample of the whale bone under study, the
X-ray diffraction and subsequent qualitative phase analy-
sis revealed the presence of hydroxyapatite (Ca₁₀(PO₄)₆OH,
ICSD PDF# 01-071-5048, space group P6₃/m). Other crys-
talline phases have not been identified. The diffraction pat-
tern in Fig. 7 shows the measured pattern as dots and the
result of the Rietveld refinement as a solid line. The refined
parameters were the lattice parameters of hydroxyapa-
tite and the degree of the {001} preferred orientation of

Fig. 4 The surface of the non-demineralized tympanic bulla under
study remained hard. Of course, an attempt to pierce such a bone with
an injection steel-based needle leads only to its noticeable deforma-
tion (a), although it leaves marks in the form of scratches on its min-
eralized surface (SEM image, c). The features of the same bone after
gentle demineralization (b) dramatically change: it becomes very soft.
This time, the sample is easily and thoroughly pierced by a similar
needle (b), leaving a characteristic mark (SEM image, d). It is worth
noting that the size and shape of the specimen prior and after demin-
eralization remains to be the same (b).
crystallites in frame of the March-Dollase model [57]. The observed anisotropic line broadening was described using the Popa model [58]. The refined lattice parameters of the hydroxyapatite from the whalebone, \( a = (9.430 \pm 0.002) \) Å and \( c = (6.897 \pm 0.001) \) Å, are in a good agreement with the lattice parameters from the ICSD PDF# 01-071-5048 data base entry (\( a = 9.425 \) Å, \( c = 6.884 \) Å).

The XRD pattern of the demineralized gray whale bone (Fig. 8) confirmed the presence of collagen. The collagen extracted from the demineralised whalebone produced the maxima of diffracted intensities at the same positions \((2\theta)\) like the reference collagen sample (Sigma-Aldrich, Germany). These diffraction maxima corresponds to a periodic arrangement of the \([\text{Pro-Pro-Gly} \text{]}_{10}\) triple helices within the structure blocks having the size of approx. \(26.9 \times 26.3 \times 20.3 \) Å\(^3\) [59–62]. The line broadening, which rapidly increases with increasing diffraction angle, is an indicator of the structural disorder of the triple helices in the structure blocks.

The ATR-FTIR analysis of native and demineralized tympanic bulla under study is presented in Fig. 9. The registered ATR-FTIR spectrum for native bone show characteristic carbonate bands \([\nu_2(\text{CO}_3^{2-})\) and \(\nu_3(\text{CO}_3^{2-})\), respectively] that appear at 870, 1409 \(\text{cm}^{-1}\) and phosphate bands \([\nu_2(\text{PO}_4^{3-}); \nu_4(\text{PO}_4^{3-});\) and \(\nu_1(\text{PO}_4^{3-}),\) respectively] that appear at 467, 558, 599, and 1010 \(\text{cm}^{-1}\) [63] and amide I band at 1644 \(\text{cm}^{-1}\) associated with presence of protein (collagen). There are three possible models of the \(\text{CO}_3^{2-}\) substitution of hydroxyapatite: A-type carbonated hydroxyapatite when the \(\text{CO}_3^{2-}\) substitutes the \(\text{OH}^-\), B-type carbonated hydroxyapatite when the \(\text{CO}_3^{2-}\) substitute the \(\text{PO}_4^{3-}\) and AB-type carbonated hydroxyapatite when \(\text{CO}_3^{2-}\) substitute both the \(\text{OH}^-\) and the \(\text{PO}_4^{3-}\) groups. The registered spectrum confirms that analyzed bone is composed of B-type \(\text{CO}_3^{2-}\) substituted hydroxyapatite (\(\text{CO}_3^{2-}\) substitutes \(\text{PO}_4^{3-}\) in hydroxyapatite) [64, 65].

The infrared spectrum registered for organic scaffold isolated by the bone demineralization using Osteosoft® show bands characteristic for collagen [66, 67], nevertheless several band or band shifts suggest that this is a collagen-lipid complex [68]. Thus, the peaks at registered 3276 \(\text{cm}^{-1}\) and 3030 \(\text{cm}^{-1}\) corresponds to Amide A and Amide B bands that are associated with N–H stretching [69] and confirming the existence of hydrogen bonds. Intense peaks registered at
2961, 2917, and 2851 cm$^{-1}$ are associated with stretching vibrations of CH$_x$ groups and indirectly confirm the presence of lipids. According to the literature, the lipid hydrocarbon tails absorb between 3050 and 2800 cm$^{-1}$ [70]. The band at 1629 cm$^{-1}$ is identified as the amide I band. Typically amide I band for collagen is observed at 1650 cm$^{-1}$. However accordingly to Liu et al. [71] a shift of amide I band from 1650 to 1629 cm$^{-1}$ is indicative of either a structural rearrangement of the existing tissue proteins or the expression of a new set of proteins with different structural characteristics.

Fig. 6 Label-free multiphoton microscopy of the demineralized tympanic bulla (red channel: CARS, green channel: TPEF, blue channel: SHG). Images were taken in a white region (a), in a yellow region (c) and in a mid-region (b) where the material was only slightly yellowish (compare with Fig. 2c). In all three regions, the mesh of collagen fibers displayed by SHG is clearly visible, as well as many cells dispersed in the extracellular matrix shown by TPEF. In the yellow regions displayed in (c) extracellular matrix other than collagen is visible as combination of CARS and TPEF signals. The arrowheads indicate structures that may be identified as the pores shown in Fig. 3.

Fig. 7 X-ray diffraction pattern of the gray whale tympanic bulla powdered sample. Open circles represent measured data points. The solid curve shows the diffraction pattern of hydroxyapatite refined to the measured data.
additionally it is highly characteristic of intermolecular hydrogen bonding. The 1541 cm\(^{-1}\) is attributed to amide II band resulting from the N–H bending vibration and from the C-N stretching vibration. While amide band is confirmed by registering peaks at 1453 and 1241 cm\(^{-1}\) \[72\]. However, peak at 1240 cm\(^{-1}\) may be also associated with antisymmetric stretching vibrations of PO\(_2\)\(^{-}\) groups’ characteristic for phospholipids \[73\]. The peak at 1399 cm\(^{-1}\) corresponds to wagging vibrations of CH\(_2\) and CH\(_3\), while 1340 cm\(^{-1}\) is attributed to CH\(_2\) side chain vibration of collagen, by some of researchers it is discussed to be marker for integrity of collagen \[74, 75\]. Absorptions at 1033 and 1079 cm\(^{-1}\) arise from the ν(C–O) and ν(C–O–C) absorptions of the carbohydrate moieties.

However, in spite of collagen domination in the organic matrix obtained after demineralization (Fig. 4b and d), the results of SDS-PAAG analysis of this fraction evidently confirm not only its presence, but also the existence of other unknown proteins (i.e. with molecular weights around 110, 70, 55, 42 and 20 kDa) (Fig. 10, line 2). Corresponding bands have not been detected in the collagen standard sample (Fig. 10, line 3). Our results from this analysis unambiguously confirm the presence of other non-collagenous proteins in the organic matrix, whose roles in the development of the hypermineralized phase of this highly specialized bone remains unknown. Accordingly, conducting a proteomic analysis of protein bands that are clearly visible on the gel presented by us (Fig. 10, line 2) seems to be the next step for identifying these components in the future studies.

Thus, our analytical results suggest the existence of collagen-lipid complex as an organic framework on which calcification within tympanic bulla of this gray whale takes place. At the same time, we assume that, in contrast to the neutrally charged collagen molecule, acidic phospholipids may be active players in the biomineralization process within tympanic bulla of whales. As reported previously \[76\], acidic phospholipids when integrated into membranes are responsible for formation of specific environments in which calcification can develop due to a sequence of initial calcium ion binding. Increasing ion concentration and further formation of (CaHPO\(_4\))\(_2\) dimers drives their condensation into a Ca\(_9\)(PO\(_4\))\(_6\) unit bound to the phospholipid-based constructs. Greater calcium phosphate clusters can be formed on such bound loci that act as nuclei for their growth and stabilization. The in vivo development of carbonated HAP can be similar to mechanisms of cardiovascular mineralization where, for example, phosphatidylserine of membranous origin is believed to complex with calcium and phosphate ions, creating a nidus for HAP crystal formation \[77\]. Thus, the next task is to identify individual compounds within isolated collagen–lipid complex and determine if they are negatively charged biomolecules of lipid origin. Of course, the question of the possible intermolecular interaction between collagen and lipids remains extremely intriguing and needs to be resolved as well.

### 4 Conclusion

In conclusion, we have demonstrated for the first time that gray whale tympanic bulla represents an example of hypermineralized bones that contains collagen-lipid complex as organic matrix. Our results underline the uniqueness of this very special case of extreme biomineralization
that occurs at very low temperatures (36.6 °C) and in the
presence of hydrophobic organic phase. At the moment,
the principle of operation of the molecular pump remains
unclear, specifically in how super-saturation with calcium
ions is achieved in a limited space of the forming tympanic
bullae. What is clear is that evolutionary development of
such hypermineralized hard tissue constructs was cru-
cial for survival of cetaceans starting on their embryonal
level. It will be necessary in the future to propose corre-
sponding calculation program for better understanding of
biomineralization kinetic parameters during the develop-
ment of whales from embryo to adult.

We also believe that unrevealing the molecular and
biochemical mechanisms of hyperbiomineralization will
allow developing strategies for the biomimetic creation
of similar composites in the laboratory, or even on large
scale using commonly and industrially available raw mate-
rials (i.e. collagens, fatty acids, calcium carbonates and
phosphates). In conclusion, we actively urge the scientific
community to support environmental and environmental initiatives to protect whales in all seas of the oceans.

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Compliance with ethical standards

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Consent to participate All of the co-workers have agreed to participate.

Consent for publication All of the co-workers have agreed to publication.

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Fig. 10 SDS-PAGE analysis of collagen isolated from whale ear bone. The proteins isolated from the whale ear bone under study have been separated using the SDS-PAGE and visualized by Coomassie G-250 staining. Loading: #1—protein marker; #2—whale ear bone proteins isolated from the demineralized specimens (see Fig. 4b, d); #3—control collagen type I standard (Sigma-Aldrich Cat. No. C9791); #4—pepsin-containing digestion buffer.

References

1. I. Polishchuk et al., Science 358, 1294 (2017)
2. G.L. Koons GL, M. Diba, A.G. Mikos Nat. Rev. Mater. (2020) https://doi.org/10.1038/s41578-020-0204-2
3. H. Ehrlich, Extreme Biomimetics (Springer, Cham, 2017)
4. H. Ehrlich, Marine Biological Materials of Invertebrate Origin (Springer, Cham, 2019)
5. R. Amils, C. Ellis-Evans, H.G. Hinghofer-Szalkay, Life in Extreme Environments (Springer, Dordrecht, 2007)
6. H. Ehrlich et al., J. Mater. Chem. B 1, 5092–5099 (2013)
7. M. Wysokowski et al., RSC Adv. 4, 61743–61752 (2014)
8. M. Wysokowski et al., Nano Res. 8, 2288–2301 (2015)
9. M. Wysokowski et al., Polymers 7, 235–265 (2015)
10. M. Wysokowski et al., Mat. Chem. Phys. 188, 115–124 (2017)
11. I. Petrenko et al., Int. J. Biol. Macromol. 104, 1626–1632 (2017)
12. I. Petrenko et al. Sci Adv. 5, eaax2805 (2019).  
13. T. Sztakowski et al., RSC Adv. 5, 79031–79040 (2015)
14. T. Sztakowski et al., Nano Res. 11, 4199–4214 (2018)
15. B. Cozzi et al., Anat. Rec. 293, 235–242 (2010)
16. P. Zioupos Eur. J. Morph. 42, 31–41 (2005).
17. Z. Li and J.D. Pasteris Am Mineral. 99, 645–653 (2014).
18. V. De Buffrenil et al., Int. J. Paleobiol. 14, 57–65 (2000)
19. J.D. Currey, J. Biomechanics 12, 313–319 (1979)
20. A. Pye, J. Morphol. 118, 495–510 (1966).
21. G. Boenninghaus, Zool. Jahrb., Abt. Anatomie 19, 189–360 (1904).
22. M. Yamada, Sci. Rep. Whales Res. Inst. 8, 1–79 (1953)
23. E.G. Ekdale, A. Berta, T.A. Demere, PLoS ONE 6, e21311 (2011)
24. E.G. Ekdale, J. Morphology 277, 1599–1615 (2016)
25. J. Schnitzler et al., Sci. Rep. 7, 46734 (2017)
26. S. Huggenberger, H. Oelschlager, B. Cozzi, Atlas of the Anatomy of Dolphins and Whales. (Academic Press, New York, 2018).
27. F.N. Schmidt et al., Proc. R. Soc. B 285, 20181820 (2018)
28. V. de Buffrenil, et al. J. Zool., Lond. 262, 371–381 (2004).
29. J.D. Sensor et al., Mar. Mam. Sci. 34, 347–364 (2018)
30. F.C. Fraser, P.E. and Purves, Bull. Br. Mu. Nat. Hist. 7, 1–140. (1960)
31. G. Behrmann, Lutra 30, 113–122 (1987)
32. R.R. Fay, Structure and Function in Sound Discrimination Among Vertebrates. In: D.B. Webster, A.N. Popper A.N.,R.R. Fay (eds) The Evolutionary Biology of Hearing. (Springer, New York, 1992).
33. D.R. Ketten, Bioacoustics 8, 103–135 (2012)
34. D.R. Ketten, The Cetacean Ear: Form, Frequency, and Evolution. In: J.A. Thomas, R.A. Kastelein, A.Y. Supin(eds) Marine Mammal Sensory Systems. (Springer, Boston, 1992).
35. S. Lees, D.B. Hanson, E.A. Page, J. Acoust. Soc. Am. 99, 2421 (1996)
36. T. Park, E.M.G. Fitzgerald, A.R. Evans, Biol. Lett. 12, 20160060 (2016)
37. B. Cozzi et al., PLoS ONE 7, e37110 (2012)
38. M. Yamato, K. Khidas, N.D. Pyenson, R.E. Fordyce, J.G. Mead, J. Anat. 228, 125–136 (2016)
39. H. Ehrlich et al., Materialwiss. Werkst. 37, 552–557 (2006)
40. H. Ehrlich et al., Micron 39, 1062–1091 (2008)
41. H. Ehrlich et al., J. Adv. Microsc. Res. 5, 100–109 (2010)
42. H. Ehrlich et al., Adv. Funct. Mater. 26, 2503–2510 (2016)
43. H. Ehrlich, P. Koutsoukos, K. Demadis, O. Pokrovsky, Micron 40, 169–193 (2009)
44. J.P. Simpson et al., J. Archaeol. Sci. 69, 29–38 (2016)
45. S.A. Dyshlovoy et al., Cancers 11, 1690 (2019)
46. S.A. Dyshlovoy et al., Mar. Drugs 18, 251 (2020)
47. S.A. Dyshlovoy, Proteomics 16, 1590–1603 (2016)
48. H.M. Rietveld, Acta Crystallogr. 22, 151 (1967)
49. L. Lutterotti, S. Matthies, H. Wenk, Comm. Power Differ. Newsl. 21, 1 (1999)
50. G. Gailey et al., Sci. Rep. 10, 1553 (2020)
51. J.A. Guerrero “Feeding behavior of gray whales in relation to patch dynamics of their benthic prey” Master’s Thesis. (1989).
52. M.S. Kerr, Dev. Biol. 20, 1–17 (1969)
53. E. Hallberg, Cell Tissue Res. 184, 45–65 (1977)

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