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Hypermineralized whale rostrum as the exemplar for bone mineral

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Abstract

Although bone is a nanocomposite of mineral and collagen, mineral has been the more elusive component to study. A standard for bone mineral clearly is needed. We hypothesized that the most natural, least-processed bone mineral could be retrieved from the most highly mineralized bone. We therefore studied the rostrum of the toothed whale *Mesoplodon densirostris*, which has the densest recognized bone. Essential to establishment of a standard for bone mineral is documentation that the proposed tissue is bone-like in all properties except for its remarkably high concentration of mineral. Transmitted-light microscopy of unstained sections of rostral material shows normal bone morphology in osteon geometry, lacunae concentration, and vasculature development. Stained sections reveal extremely low density of thin collagen fibers throughout most of the bone, but enrichment in and thicker collagen fibers around vascular holes and in a minority of osteons. FE-SEM shows the rostrum to consist mostly of dense mineral prisms. Most rostral areas have the same chemical-structural features, Raman spectroscopically dominated by strong bands at $\sim 962\text{ cm}^{-1}$ and weak bands at $\sim 2940\text{ cm}^{-1}$. Spectral features indicate that the rostrum is composed mainly of the calcium phosphate mineral apatite and has only about 4 wt.% organic content. The degree of carbonate substitution ($\sim 8.5\text{ wt.}\%$ carbonate) in the apatite is in the upper range found in most types of bone. We conclude that, despite its enamel-like extraordinarily high degree of mineralization, the rostrum is in all other features bone-like. Its mineral component is the long-sought uncontaminated, unaltered exemplar of bone mineral.

Keywords

bone mineral; rostrum; apatite; histology; scanning electron microscopy; Raman spectroscopy

Introduction

The typical human femur consists of about 55 wt.% mineral, 30 wt.% collagen, and 15 wt.% water [1], which means volumetrically sub-equal amounts of collagen and mineral. Both the mineral and collagen components are nanometer in scale, making bone truly a nanocomposite. Whereas natural samples of almost pure or easily isolated collagen (specifically, collagen I) are readily available, the same cannot be said of the mineral (carbonated apatite) component of bone. Historically, those who wanted to investigate the

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mineral component of bone studied synthetic apatite analogs or used aggressive chemicals (e.g., ethylenediamine, sodium hypochlorite, or hydrazine) to selectively dissolve the collagen from bone, thereby isolating the mineral as a residue [2–7]. Increasing evidence, however, indicates that both chemical (e.g., concentration of carbonate and acid phosphate) and physical properties (e.g., degree of crystallinity and crystallite size) of the bone mineral can be changed during removal of collagen [8,9]. In recent years, nondestructive spectroscopic techniques, such as Raman and IR spectroscopy, have been used to obtain separate spectral information on the mineral and the collagen components *in situ* in bone samples, typically with little to no chemical preparation of the sample [10–15]. However, the presence of collagen does complicate the interpretation of vibrational spectra [9], and such techniques do not permit detailed chemical (elemental) analysis or mechanical testing of the bone mineral. The ideal sample for chemical, mechanical, and additional tests would be bone that naturally consists almost entirely of mineral.

The rostrum of the Blainville's beaked whale (family Ziphiidae), more formally known as the toothed whale *Mesoplodon densirostris*, contains the densest bone so far recorded. According to Zylberberg et al. [16], the snout-like rostrum has a density of 2.6 g/cm³, and Rogers and Zioupos [1] reported that the rostrum has up to ~96 wt.% mineral, with the remainder mostly collagen. Its extremely high density and mineral content explain why it is the mechanical properties of the hypermineralized rostrum that have been most studied. It was reported that the rostrum of *M. densirostris* has a Vickers hardness of ~223, Young's modulus up to 46.9 GPa, and bending strength up to 59.0 MPa in the longitudinal direction, which makes it the stiffest and hardest of all bones [17,18]. Several potential functions, e.g., to aid in echolocation, combat, and deep diving, have been discussed in previous literature [19–23]. However, the exact biological purpose of this hypermineralized material, which occurs only in the adult males, is still unknown.

The ziphiid rostrum is often modified compared to that of other toothed whales, i.e., the thickened vomer usually fills the mesorostral groove [21,23–25]. Although many aspects of the rostrum of this whale are shared by most of the members of the beaked whale group, in *M. densirostris*, the vomer, together with the maxilla and premaxilla, are all hypermineralized [21,26]. The dense rostrum is composed dominantly of remodeled tissue [1,16,21,27], whereas some other beaked whales that do not have hypermineralized rostra, e.g., *Aporotus recurvirostris*, have almost no remodeling in this tissue [26]. The hypermineralized rostrum also differs from the somewhat less hypermineralized otic region of the whale, i.e., the bulla, whose hypermineralization arises through substitution of primary osteons for spongiosa [28]. Carbonated hydroxylapatite is the mineral phase in both normal bone tissue [3–6,29–37] and the rostrum [16]. As in normal bone, the *c* axes of the bioapatite crystallites are parallel to the long axes of the collagen fibrils in the rostrum of *M. densirostris*; both the fibrils and the *c* axes are also aligned parallel to the longitudinal direction of the rostrum [1,16,27].

The unusually high concentration of mineral in the adult male's rostrum -- together with its apparently normal bone features such as secondary osteons, lacunae, and organic matrix [16,18,21] -- seem to make the rostrum the ideal material in which to study the mineral phase of bone. The aim of this paper is to confirm the bone-like features (such as osteons and lacunae) of the rostrum, to investigate the tissue's microstructure (such as mineral prisms and collagen-mineral texture), and to evaluate how appropriate its mineral component is as the model for "typical bone mineral."

Materials and Methods

Materials

Three sets of rostrum samples from *M. densirostris* were studied: One is an irregular 6 mm thick prism from the skeleton of a modern adult male (#1922-143) housed in the Muséum National d'Histoire Naturelle in Paris, France, which will be referred to here as MNHN. This rostrum has been previously studied and reported on [16,17,18,21]. The second sample is a sawn prism (2.5×1×0.4 cm) from material previously investigated by Rogers and Zioupos [1], which will be referred to as KR. The KR and MNHN samples are derived from the same source, but we are uncertain of their exact spatial relation with respect to each other in the original rostrum. The third sample is material cored (2 cm long and 4 mm diameter) and studied for the first time from the rostrum of a modern adult male (#571379) housed in the Smithsonian Institution in Washington, D.C, which will be referred to as USNM. The USNM whale (405 cm long) was collected from the beach at Rodanthe, North Carolina (United States) in 1990 and since then housed in the Smithsonian Institution (no chemicals were used to preserve the rostrum).

Three additional samples were studied for purposes of comparing and contrasting the whale rostral tissue with normal bone and other mineralized, but non-osseous tissue. In recognition of the size of the whale, bone samples were selected from larger mammals, rather than from mice or rats. The chosen non-osseous tissue was dental enamel, in recognition of the fact that the ratio of mineral to organic matrix in enamel is almost exactly the same as that in the rostrum [1]. No chemicals were used to preserve these three tissues. Samples of lamb femur (local butcher) and elk antler (local collector) were investigated as bone comparisons. Enamel from the molar of a coyote (local collector) was prepared as the non-osseous sample. No orthopedic disease was identified in any of the three samples. The lamb was 1.5–2 years old. The exact ages of the other two host animals are unknown. However, the antler of the elk was selected because such tissue must be less than one year old.

Sample Preparation

Four different sub-samples were made from the MNHN and KR sawn rostrum prisms: (1) Both 15µm- and 30µm-thick transverse sections were made for optical microscopy study (prepared by Applied Petrographic Services, Inc., PA) from the MNHN prism. A portion of the KR prism was immersed in an epoxy-filled ring, allowed to set, and cut by an Isomet low-speed diamond saw (Buehler LTD, Lake Bluff, IL) for preparation of the following thin sections: (2) Millimeter-scale particles for SEM analyses were collected as breakage fragments during the sawing process. (3) A transverse cross-section (for back-scattered electron imaging) and (4) a longitudinal cross-section (for Raman microprobe spectroscopy) of a portion of the KR prism were sawn and fixed with epoxy or superglue onto a glass plate. They were thinned to several tens of micrometers thickness, ground, and polished to the level of 1 µm diamond powder. None of the materials in these MNHN and KR samples underwent histologic staining.

The cored rostral material (USNM) was studied in four types of histologic stained sections (Picrosirius Red, H&E, Goldner's Trichrome, and Nuclear Fast Red) prepared at the School of Medicine, Washington University in St. Louis. The selected cored material was embedded in paraffin and then infiltrated within 30% sucrose for 1 week to help soften the tissue. In addition, surface decalcification was carried out via EDTA to enable preparation of sections five micrometers thick. For comparison, Picrosirius Red and H&E stained sections of the elk antler and lamb femur were prepared by following the same procedures as for the stained sections of the rostrum.

Coyote molar enamel, synthetic standard hydroxylapatite (99.999% metal basis, Sigma-Aldrich®, St. Louis, MO), deproteinized lamb femur, and deproteinized elk antler, also were prepared for comparative Raman analysis. The lamb femur and elk antler samples were soaked in sodium hypochlorite (5.25%) while in an ultrasonic cleaner for 1 hour in order to remove fluorescence-causing, organic components, including collagen, from the samples' surfaces. Finally, they were washed in tap water and air-dried before Raman analysis.

Instrumentation

For all unstained sections, optical microscopy was performed using a Leitz Wetzlar (Germany) petrographic microscope. All stained sections were imaged with an Olympus BX51 microscope outfitted with an Olympus DP70 camera system.

Back-scattered electron (BSE) imaging was performed with a JEOL JXA 8200 Superprobe in the Department of Earth and Planetary Sciences at Washington University in St. Louis (MO), operated with an accelerating voltage of 15 kV. The section was sputter coated by carbon before analysis.

Field-emission scanning electron microscopy (FE-SEM) was applied using an FEI NOVA 2300 system (FEI, Hillsboro, OR) at the Nano Research Facility at Washington University in St. Louis, operated with an accelerating voltage of 5–20 kV. The rostrum particles and coyote enamel were sputter-coated by gold in a Cressington 108 sputter coater before FE-SEM imaging.

The longitudinal polished thin section made without epoxy and fully cleaned of superglue was studied by confocal Raman microprobe spectroscopy. Analysis was performed with a fiber-optically coupled Raman microprobe (HoloLab Series 5000 Raman Microprobe, Kaiser Optical System, Inc.). The 532 nm excitation was delivered by a frequency-doubled Nd:YAG laser, which was coupled to a Leica microscope (Germany) with an ultra-long-working-distance MSPlan 80×objective, N.A. = 0.85 (Olympus, Japan). The spectral region of 100–4000 cm^{-1} was recorded with a spectral resolution of 2.5 cm^{-1} . The power of the incident laser was 10 milliwatts as measured at the surface of the sample. Intensity, wavelength, and Raman shift position were calibrated based on a NIST secondary standard, gas emission lines, and a laboratory standard. Reproducibility of the Raman shift position for a silicon wafer was $520.5 \pm 0.1 \text{ cm}^{-1}$. The typical acquisition time per analysis spot (~1 μm diameter) was 32×4 seconds. Spectra were acquired using Kaiser Optical's Holograms^R software.

Spectral Deconvolution

The region between 900 and 1000 cm^{-1} was selected for deconvolution of the peak at ~962 cm^{-1} . Deconvolution yielded one strong band at ~962 cm^{-1} and a weaker band at ~948 cm^{-1} , which are both possible $^{12}\text{P-O}$ stretching modes according to group theory [38]. The ~962 cm^{-1} peaks in spectra of enamel and standard hydroxylapatite were best fit by using only a single band. In the peak area ratios presented in this paper, the area of the ~962 cm^{-1} peak is defined by the sum of both (if present) of its deconvolved bands. All the acquired spectra of bone materials were processed using Grams/32^R software (Galactic, Salem, NH). Baseline correction was carried out by deriving a polynomial fit to manually selected points chosen so as to avoid distortion of the peaks. The same procedure was applied to all samples. The bands for the peak at ~962 cm^{-1} were fit based on a mixed Gaussian-Lorentzian algorithm after baseline correction. The deconvolution was processed three times for each spectrum and all the correlation coefficients for band fitting were > 0.999.

Results

Optical microscopy of unstained and stained sections

The unstained sections show a banded color distribution in the longitudinal section under transmitted, plane-polarized light: parallel, discontinuous black- and brown-banded areas on a millimeter length scale (Fig. 1A). In addition, some very dark narrow curvilinear features exist (see arrows in Fig. 1B). Black linear features in many cases seem to be fractures, but some are collagen-rich as discussed in the following Raman section. One prominent, through-going hole in the transverse section has a diameter of about 400 μm (Fig. 1B) and appears to be the channel of a large vascular structure.

The transverse section shows a distinctly different micro-morphology. Instead of lamellar bands, there are numerous normal-appearing osteons with a diameter ranging from 150 to 300 micrometers (Fig. 1C), the sizes of which are similar to those studied by Lambert et al. in other beaked whales with hypermineralized rostra [21]. These are secondarily developed osteons [16,21,26,27] with a haversian canal approximately at the center of each. However, these osteons do not show obvious internal concentric banding, unlike normal bone. The relative (compared to the longitudinal section) abundance of approximately circular, through-going holes indicates that many of the prominent vascular canals, in addition to the haversian canals, are oriented parallel to the longitudinal section of the rostrum (Fig. 1C). Abundant lacunae appear in both longitudinal and transverse sections (speckling in Fig. 1A, B, and C) and exhibit a major axial length of ~ 10 micrometers (see arrows in Fig. 1D). The back-scattered electron image in Figure 1D shows a homogeneous elemental distribution (same gray-tone) in the rostrum's transverse section, with only small variations (darker gray, indicating lighter elements; inferred presence of collagen) in the area around the large vascular hole (Fig. 1D).

All four types of stains revealed a strongly bimodal distribution of collagen in the rostrum, i.e., regions of enrichment in collagen appearing as deeply stained isolated regions within the dominant, lightly stained, collagen-poor matrix. In both cross-polarized (Fig. 2A and B) and plane-polarized light (Figs. 2C, D, and F), deeply stained osteons are randomly distributed within the sections.

In the transverse section (see Fig. 2A), cross-polarized light revealed round to elliptical features stained yellow-red, representing thick collagen fibers around osteons and other vascular holes. Figure 2B shows a longitudinal section in cross-polarized light, displaying a lower population density of vascular holes than the transverse section. Thin collagen fibers (green) dominate the section and are approximately parallel to the longitudinal direction, which was also confirmed by TEM images [16,27]. Therefore, the volumetrically dominant "typical" areas of the rostrum have spatially extensive collagen, but its concentration is extremely low. Various stains produce almost the same distribution of coloration (see Fig. 2C&D and Fig. 2E&F), which confirms the enrichment of collagen within certain osteons and around larger vascular holes. These enrichments contrast with the obviously very low collagen concentration in most osteons, as noted by arrows in Fig. 2D. Thus, the bimodality of over-all tissue types indicated above extends to the osteon level: One group of osteons is deeply stained; the other shows no or extremely light stain (Fig. 2C and D). The stained group is present as isolated entities or in clusters (Fig. 2C, D). The diameters of Haversian canals of both the high-collagen and low-collagen osteons are variable.

In many cases within the strongly stained osteons of the rostrum, the color is heterogeneously distributed, appearing especially dark on the outermost edge of the osteon (Fig. 2F), e.g., osteon 1 in Fig. 2F, and the dark (stain-enriched) outermost edge is connected to the central holes through small channels. In Fig. 2F, osteons 1 and 2 appear perpendicular

to the transverse section, but the slight ellipticity of the Haversian canal of osteon 3 suggests non-perpendicularity. Therefore, there are slight variations in the growth directions of osteons.

In contrast, the elk antler and lamb femur sections are almost homogeneously deeply stained; the osteons are closely packed and homogeneously distributed (Fig. 3D and G). In the cortical bone of the elk antler and lamb femur (Fig. 3F and I), the osteons internally show a concentric distribution of stained collagen bundles, which appear as alternating light and dark lamellae under crossed-polarized light. However, for the rostrum, individual lamellae typically are almost indiscernible. We interpret the middle ring of darkness in the otherwise bright osteons in Figure 3C to reflect the dearth of collagen (seen by the lightness of the stain in Figure 3A) in this portion of the osteons. The transverse section of the rapidly grown elk antler also shows several red vessels associated with each osteon (Fig. 3E and F). In addition, the outermost zone of some osteons in the elk antler is connected to the Haversian canal through these small red vessels, as seen in Fig. 3C and analogous to previous discussion of Fig. 2F. In the transverse section of the lamb femur, the osteons are dominantly arranged longitudinally. No large vascular holes (see Fig 2F and Fig. 3C) were identified in the cortical bone of the lamb femur or the elk antler.

Scanning Electron Microscopy

The rostrum is composed of dense mineral prisms as seen in FE-SEM images (Fig. 4). The elongated prisms, apparently consisting of plate-like nanocrystals, have irregular shapes with lengths of 1–2 μm and widths of several hundred nanometers (Fig. 4A). The rough surface formed by such prisms of organized mineral bundles in the rostrum can be distinguished, at similar magnification, from surfaces of other bone materials that contain bundles of collagen [39–43]. No collagen fibrils were discovered by SEM among the mineral prisms in the rostrum, presumably due to their rarity and thinness. The mineral prisms aligned approximately along the longitudinal direction, however, show a slight variation in their orientation, as revealed in Fig. 4A.

Figure 4B shows an osteocytic lacuna with a diameter of $\sim 10 \mu\text{m}$ exposed on a fractured surface. The size is very close to that of lacunae in, for instance, human, rabbit, and bovine femora [3,42–44], but the inside surface of the rostral lacuna has no texture indicative of collagen fibers. Openings for canaliculi radiating from lacunae indicate a 3-dimensional vascular system (cf. [17]).

A comparison between the non-deproteinized rostrum and coyote enamel, both representing material with about 96 wt.% mineral [1], shows a similar prismatic structure but distinctive morphologies and sizes of the mineral prisms and their constituent platelets. At the micrometer scale (Fig. 5A and C), the mineral prisms in the rostrum are much smaller than those in coyote enamel, e.g., one rostrum prism shows a length of 3.5 μm , compared to the much larger 22.0 μm of one prism in the coyote enamel. However, at the nano-meter scale, the elongated mineral platelets in the rostrum (Fig. 5B) are universally wider than those in the coyote enamel (Fig. 5D): platelets within two randomly selected prisms show a width of 225 nm vs. 74 nm, respectively. The irregular edges within the prisms (see rectangular area in Fig. 5B) presumably indicate a stack of several thin platelets that have broken.

Raman Microprobe Spectroscopy

Raman spectroscopy has been used widely to investigate the mineralogy of and to document the collagen in bone materials [10,11,13–15,38,45–48]. The most characteristic spectral regions of typical bone materials are 400–1200 cm^{-1} for mineral bands and 2500–3150

cm^{-1} for organic bands. In principle, the ν_1 P-O symmetric stretch of phosphate at $\sim 962 \text{ cm}^{-1}$ is the major indicator of apatite, including bioapatite in bones [10,13–15,38,45–48].

The longitudinal section of the undecalcified, unstained, non-deproteinized, rostrum, despite the appearance of different colors in transmitted light under the optical microscope, shows very similar Raman spectroscopic features among 53 randomly analyzed points (see the spectrum of a typical area of rostrum in Fig. 6). All of the spectra are dominated by peaks at $\sim 962 \text{ cm}^{-1}$, with one randomly selected lacuna in the rostrum showing slight enhancement of organic components compared to the typical rostrum (Fig. 6).

The $\sim 1003 \text{ cm}^{-1}$ band characteristic in bone, which is caused by C-C aromatic ring vibrations of the phenyl group [13,15], is absent in spectra of the highly mineralized rostrum (see typical area in Fig. 6). It is likely below our detection limit due to the low concentration of collagen.

All 53 spectra have a prominent feature at $\sim 1073 \text{ cm}^{-1}$, which is the indicator of carbonate that is structurally incorporated within apatite [13,15,46,48]. In principle, the carbonate ion substitutes either in the phosphate tetrahedron (B-type) or in the hydroxyl site (A-type). Biological (i.e., low-temperature) apatites are dominated by the B-type carbonate substitution [5,32,34,35,48,49], for which the known band position is $\sim 1073 \text{ cm}^{-1}$ [13,14,46,48].

The ratio of the area of the $\sim 1073 \text{ cm}^{-1}$ band to the area of the $\sim 962 \text{ cm}^{-1}$ band(s) is a quantitative indicator of the degree of carbonation of apatite [45,50]. The average value of that ratio in our 53 studied spots is 0.302 ± 0.033 (Max: 0.403, Min: 0.226), which indicates that the degree of carbonation of the apatite is quite homogeneous (± 10 relative%) throughout the rostrum (Fig. 7). Our group has calibrated that ratio based on a suite of synthetic carbonated apatites of independently determined carbonate concentration. Based on that calibration [46], the bioapatite component of the rostrum averages $8.5 \pm 0.8 \text{ wt. \%}$ carbonate (Max: 11.0 wt.%, Min: 6.6 wt.%).

Several apatitic materials were analyzed for specific comparison with rostral bioapatite: enamel, as another hypermineralized (96–97 wt.% mineral) bioapatite material [51]; lamb femur, as a typical mammal bone; elk antler, as extremely rapidly formed bone (antler completely formed in < 1 year); and synthetic hydroxylapatite, as an uncarbonated apatite.

Various features of the spectra yield specific structural-chemical information. The position of the ν_1 P-O stretch is sensitive to chemical substitutions in apatite. The $\sim 962 \text{ cm}^{-1}$ peak position in bone materials is higher than the position in enamel but lower than in standard hydroxylapatite (Fig. 8 and Table 1). Among bone materials, the rostrum has a slightly higher ν_1 P-O peak position than the elk antler and lamb femur. The slight upshift in the ν_1 P-O peak position in bioapatite often indicates some substitution of fluoride for hydroxyl [47]. All of the bone materials have a strong peak at $\sim 1073 \text{ cm}^{-1}$, indicating that bone apatite contains more carbonate than does enamel. The normalized intensity of the $\sim 1073 \text{ cm}^{-1}$ peak in the rostrum indicates that the latter has about the same degree of carbonate substitution as the elk antler, but distinctly less than the lamb femur.

Widths of Raman bands indicate the degree of atomic order in the material; the wider the band, the more atomically disordered the material is. The full width at half intensity of the $\sim 962 \text{ cm}^{-1}$ band is considerably larger in bone and tooth materials than in standard hydroxylapatite (5.4 cm^{-1}). The band width in the rostrum is 14.8 cm^{-1} , which is slightly narrower than the $\sim 15.5 \text{ cm}^{-1}$ in deproteinized elk antler and lamb femur but considerably wider than the 13.0 cm^{-1} in non-deproteinized enamel (Table 1). Thus, bone apatite is atomically more disordered than enamel or synthetic hydroxylapatite, and the rostrum

apatite is slightly more ordered than that of the lamb femur and elk antler, but distinctly less ordered than tooth enamel.

In the evaluation of crystallite size in bioapatite materials, an alternative to direct measurement via FE-SEM is the use of X-ray diffraction (XRD). A previous comparative XRD study of the rostrum showed narrower peaks for its apatite, indicating somewhat larger crystals (based on the Scherrer equation, after accounting for internal crystal strain) than in normal bones [1]. The greater degree of atomic order in the rostrum crystallites compared to that in normal bone, as inferred from Raman peak widths, is presumably related to the larger size of the rostrum crystals. Both our Raman peak widths (Table 1) and Rogers and Zioupos' XRD peak widths (Figure 2 in [1]) show the rostrum crystallites to be distinctly different from (less atomically ordered and smaller than) those in enamel.

The greatest spectral contrast within typical rostral material is shown by some narrow, black curvilinear features, e.g., to the left of the vascular hole in Fig. 6, which are so enriched in organics that their Raman analyses resemble those of normal bones. In the spectrum of such a linear feature, the $\sim 1003\text{ cm}^{-1}$ peak of collagen was detected, whereas it is absent in typical areas. The additional peaks at ~ 1155 and $\sim 1517\text{ cm}^{-1}$ match the spectral features (enhanced by resonance with the 532 nm excitation laser) of carotenoids in blood plasma, as reported by Rein et al. [52]. One additional peak at $\sim 1453\text{ cm}^{-1}$ can be assigned to the (CH_2 , CH_3) modes typically identified in bone [15,48]. Normal bones have a significant organic content, up to about 35 wt.% in the mineral-organics-water system [1,33], dominated by type I collagen. Many of the Raman bands in the spectral region $2500\text{--}3150\text{ cm}^{-1}$ do not permit specific identification as “collagen,” but rather only general assignment to the organic components. The (CH_3) symmetric and (CH_2) asymmetric stretches occur at $\sim 2940\text{ cm}^{-1}$. These peaks in bone samples are normally assigned to vibrations within collagen, but also are expressed by other organic components such as noncollagenous proteins [10,48,53].

The ratio of the area of the deconvolved $\sim 2940\text{ cm}^{-1}$ band components to the area of the $\sim 962\text{ cm}^{-1}$ band components provides information on the collagen/mineral ratio in the bone. Previous study in our laboratory permits a one-point calibration between spectral ratios and organic content: Fischer rat bones yielded a Raman ratio of 2.551 (1/0.392) [54], and subsequent ashing showed the bones to contain 27 wt.% organic components and 73 wt.% mineral [55]. Hence, estimated mineral contents in the present study are 96 wt.% for the typical area and 55 wt.% for the collagen-rich area, whose spectra are shown in Figure 6.

Discussion

The hypermineralized rostrum has histological features analogous to those of normal bone, e.g., in the abundance, size, and shape of its osteons, lacunae, and vascular holes. Confocal Raman microprobe spectroscopy, which analyzes the surface of the sample, indicates the hypermineralized regions (i.e., typical areas) of the rostrum to be quite homogeneous in mineral abundance and mineral composition. The homogeneity of the dominant, hypermineralized regions is also confirmed by BSE images.

Stained, decalcified longitudinal and transverse sections reveal a previously unreported dichotomy in tissue types. In addition to the typical areas of hypermineralization, there are small regions of high collagen content, in which the abundance of collagen is equal to or greater than that throughout the elk antler and lamb femur. These organic-rich areas also contain much of the vasculature. In addition, Raman spectra of the organic-enriched areas show a slightly higher band position for the ν_1 P-O stretch than does typical rostral bone, suggesting a slightly different mineral composition. These variations in mineral vs. collagen

content and in mineral composition are possibly an adaptation of very brittle bone material to the encasement of very compliant blood vessel walls.

The reason for the remarkably “normal” bone-like appearance of the hypermineralized regions is histologically demonstrated by the homogeneous distribution of collagen -- although as thin fibers in low concentration. Our stained sections complement the TEM images of decalcified rostrum shown by Zylberberg et al. [16]. Moreover, even the less abundant, strongly stained osteons are only rich in collagen toward their outer edges and directly around their Haversian canals, and those zones are connected by small channels or vessels. It is indeed the depletion in collagen within the central zones of the stained osteons that accounts for their lack of distinguishable alternating bright and dark lamellae under crossed polarizers -- an alternation that is distinctive of normal bone such as the elk antler and lamb femur. Lambert et al. [21] also noted a similar lack of alternating bright and dark lamellae in the osteons of fossil rostral samples from *Choneziphius planirostris* as well as *M. densirostris*.

The relationship, for instance in timing, between the hypermineralized and the more collagen-rich regions of the rostrum is difficult to determine in our samples. The osteons in the hypermineralized region are typically separate from one another. In contrast, the organic-rich osteons typically are clustered and show overprinting of two or more generations. No areas, however, were observed in which osteons of the two different types of tissue overprinted each other. The collagen-rich osteons therefore appear to still be biologically active, whereas the dominant hypermineralized areas do not show such activity. Based on histologic evidence of the overprinting of hypermineralization on normal bone tissue in a rostrum sample they studied, Zylberberg et al. [16] concluded that hypermineralization occurred during remodeling. The absence of such overprinting in our samples suggests that they were derived from a more mature animal, in which all evidence of the primary bone had been removed, except for the collagen-rich osteons associated with the vasculature. This maturation process and the bone textures it produces are distinct from those in the hypermineralized otic region in whales [28] and osteopetrosis in humans [31]. The fact that vascularization typically develops before mineralization of bone tissue suggests that the regions of present high collagen content may have been preserved from the effects of hypermineralization.

The appearance under FE-SEM of the broken surface of the rostrum is very different from that of average mammalian cortical bone, in which mineral nano-platelets and collagen are so intimately intermixed as to make it difficult to define the size, shape, and organization of the mineral. In contrast, mineral prisms and elongated platelets, which are readily visualized in the rostrum, are tightly packed and aligned approximately along one direction, i.e., most probably the longitudinal direction. Furthermore, the Picrosirius Red-stained longitudinal section (Fig. 2B) confirms the sparseness as well as the longitudinal arrangement of the thin collagen fibers. These observations indicate that collagen, despite its extremely low concentration, could direct the growth of the mineral prisms and the platelets that constitute them. The variation in the orientation of the mineral prisms (Fig. 4A) and the slight tilting of osteons (e.g., osteon 3 in Fig. 2F) indicate that mineral and osteons in rostrum have a “quasi-scaffold” structure similar to that in enamel [56], but with a slight degree of orientational variation. The latter probably improves the mechanical properties of the rostrum.

Conclusions

We used a combination of optical microscopy of stained sections, field-emission SEM, and Raman microprobe spectroscopy to show the rostrum to be true bone tissue and to demonstrate how its extreme depletion in collagen makes it ideal as the standard for bone

mineral. Our study differs from previous work on the rostrum, which was directed toward issues of comparative anatomy and mechanical properties of bone. In distinction from earlier studies of the rostrum, our research (1) provided side-by-side comparisons between the rostrum and normal bones, (2) applied Raman microprobe spectroscopy to characterize the rostrum, (3) highlighted the dichotomy in collagen concentration within the rostrum by means of stained sections and backscattered electron images, and (4) applied high-resolution (field-emission) FE-SEM, rather than standard lower-resolution SEM, to investigate the orientation (and the variation in the orientation) of mineral prisms in the rostrum. This also is the first study to make a comparison between enamel (equally hypermineralized tissue) and the rostrum at different spatial scales. Our Raman and FE-SEM data demonstrate that, despite the hypermineralized rostrum's similarity to tooth enamel in its degree of mineralization, the rostrum's mineral component is structurally-chemically different from it and instead distinctly bone-like.

This is the first time in the past decade that rostrum tissue has been featured in a medical journal. We suggest that more, clinically pertinent information could be gleaned from this tissue especially as regards the control that collagen exerts over mineral deposition. The rostrum may be a useful comparator for hypermineralization states of human bone associated with skeletal fragility, such as osteopetrosis, or acquired states such as through bisphosphonate treatment. As an "extreme" [1,18] example of bone tissue, the rostrum still has more to offer the medical community.

In summary, most of the rostrum of the adult male *M. densirostris* is an extremely highly mineralized bone material, ~96 wt.% of which is carbonated hydroxylapatite. This is the approximate level of mineral concentration found in normal bone that has already undergone two or more cycles of likely mineral-altering hydrazine deproteinization [8]. With the exception of its unusually high mineral content, however, the rostrum is typically bone-like in its histology and in the spectral features of its mineral. The extremely reduced content of organic matrix in the rostrum makes mineral analysis, e.g., by Raman spectroscopy and high-resolution SEM, feasible and accurate without the analytically questionable processes of deproteinization. We propose that the unprocessed rostrum is an ideal example of "bone mineral." This material therefore is very appropriate for future study of the detailed chemistry and mechanical properties of bone mineral, which are presently under investigation.

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Fig. 1. Unstained, undecalcified, non-deproteinized MNHM (A, B, and C) and KR (D) rostrum sections under plane-polarized transmitted light A: Overview of 30 μ m-thick longitudinal section; B: Large vascular hole in 30 μ m-thick unstained transverse section. The arrows indicate some curvilinear features; C: Overview of 15 μ m-thick unstained transverse section; D: Back-scattered electron image of transverse section. Several lacunae are indicated by arrows.

Fig. 2.

Transmitted-light images of the stained histological sections made from decalcified USNM rostrum. Transverse (image A) and longitudinal sections (image B) stained with Picrosirius Red show thick collagen (yellow-red) and thin collagen (green) under cross-polarized light. The arrow in B indicates the longitudinal direction. C: Enlargement in plane-polarized light of the rectangular area in image A, stained by Goldner's Trichrome. D: Same area as image C, stained by Nuclear Fast Red. The arrows in D indicate collagen-poor osteons with very little stain. E: Transmitted, cross-polarized light image of the rectangular area in image C stained by Picrosirius Red. F: Same area as in image E, but in plane-polarized light and stained with H&E, showing three osteons (O) and an apparent vascular hole (VH) of larger diameter.

Fig. 3.

H&E (plane-polarized) and Picrosirius Red (cross-polarized) stained transverse sections of the decalcified whale rostrum (A, B, and C), central cortical region of elk the antler (D, E, and F), and central cortical region of the lamb femur (F, H, and I). The arrows in A indicate almost unstained osteons. The arrows in C and F show channels or vessels connecting central holes to outermost areas enriched in collagen. Images C, F, and I are enlargements of the rectangular areas within images B, E, and H, respectively. The exposure time for all H&E sections is 1/1800 second, and the exposure time for all cross-polarized light images is 1/3.5 s. All scale bars are 500 μ m.

Fig. 4. FE-SEM images of the natural fracture surface of the non-deproteinized rostrum: A: Elongated mineral prisms in the typical area are tightly compact. The region in the square illustrates slight variations in the orientation of the prisms. B: One lacuna with its connecting canaliculus system. Openings of canaliculi can be seen inside of the lacuna (arrows).

Fig. 5.

Comparison of FE-SEM images of the rostrum (A & B) and coyote enamel (C & D): A: Elongated mineral prisms in a typical area are seen along a crack. One representative prism shows a length of 3.5 μm (range: 1–4 μm). B: At the nanometer scale, one elongated platelet in the rostrum shows a width of 225 nm (range: 190–270 nm). C: One typical prism in coyote enamel shows a length of 22.0 μm (range: 18–25 μm). D: At the nanometer scale, one elongated platelet in coyote enamel shows a width of 74 nm (range: 70–100 nm). Both the rostrum and enamel are non-deproteinized samples.

Fig. 6. Representative Raman spectra of a typical area, a lacuna, and a collagen-rich curvilinear feature in the longitudinal section of the non-deproteinized rostrum. Several diagnostic peaks have been indicated in the spectrum of collagen-enriched bone: mineral (apatite) bands are marked A, and collagen bands are marked C. Images of lacunae (A) and one collagen-enriched curved feature (B) are shown under reflected light (scale bar =100 μm).

Fig. 7. Ratios of the area of ~1073 cm⁻¹ band to the area of ~962 cm⁻¹ band for 53 spectra. Error bars show the standard deviation for three replicate deconvolutions of individual spectra.

Fig. 8.

Representative Raman spectra of rostrum, cortical bone of elk antler, cortical bone of lamb femur, coyote enamel, and synthetic standard hydroxylapatite. All spectra have been normalized in the intensity of their ~962 cm^{-1} peak and stacked after baseline correction. The samples of elk antler and lamb femur are deproteinized.

Position and full width at half intensity of the ~962 cm⁻¹ deconvolved band component (see Fig. 8) in the rostrum, elk antler, lamb femur, coyote enamel, and synthetic standard hydroxylapatite (N=5 for all data).

Table 1

	Rostrum	Elk Antler	Lamb Femur	Coyote Enamel	Standard Hydroxylapatite
Position(cm ⁻¹)	962.0±0.1	960.6±0.1	961.0±0.1	961.0±0.1	962.3±0.1
Width (cm ⁻¹)	14.8±0.1	15.4±0.1	15.6±0.1	13.0±0.1	5.4±0.1