On the pathway of mineral deposition in larval zebrafish caudal fin bone

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1. Introduction

A long standing question in vertebrate biomineralization concerns when, where and in which form the first bone mineral is deposited along the pathway leading from the environment through the tissues to the final deposition site as carbonated apatite crystals. The modes of uptake of calcium, phosphate and carbonate ions necessary for vertebrate bone formation are diverse, reflecting the different habitats in which the organisms live. In teleost fish, water is presumably the major source of these ions [1]. The distribution of ions within the organism involves the circulatory system. Vertebrate blood and other body fluids are saturated with respect to carbonated hydroxyapatite, the mineral deposited in the skeleton [2]. Ectopic mineral deposition in soft tissues is prevented by the presence of proteins such as fetuin, the mineral deposited in the skeleton [2]. Ectopic mineral deposition to the extracellular space where bone forms [9,11,12]. In adult zebrafish

Abbreviations: ACP, amorphous calcium phosphate; OCP, octacalcium phosphate; dpf, days post fertilization; SEM, scanning electron microscopy; XRF, X-ray fluorescence.

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living larvae (Fig. 1a). Tail fin bone begins to develop at 17 days post-fertilization (dpf) and is fully formed by 30 dpf [36]. During this time, 18 fin bones are formed. The basic anatomy of the developing bone during the early stages is similar to the mature zebrafish ray anatomy described above [34,37]: two half cylinders with an artery (capillary) located in the middle of each bony-ray, and veins located between the bones (Fig. 1b–d). We used the fluorescent dye calcein, together with transgenic reporters, to monitor the calcium transport pathway and the distribution of calcium mineral-containing deposits in the tissue. Calcein is known to bind calcium [38,39] and to stain the newly formed bone surface [36] (Fig. 1a). We took advantage of the fact that up to 35 dpf, zebrafish larvae take up calcein directly from the water.

2. Materials and methods

2.1. Zebrafish husbandry

Adult and larval zebrafish were raised and maintained as previously described [40] and were handled according to the guidelines of the Weizmann Institute Animal Care and Use Committee. Embryos were generated by natural spawning and raised in egg water in a 28 ± 0.5 °C incubator. Three transgenic fish lines were used in this study: Tg(ffi1:EGFP)y1 embryos expressing EGFP in endothelial/hematopoietic cells [23], were crossed to nacre mutants [41] to generate transparent animals enabling live imaging of juvenile stages (20–30 dpf); Tg(ffi1: DsRed) [42] expressing DsRed in endothelial/hematopoietic cells; Tg(oscmCherry) expressing mCherry under the regulation of the osterix promoter [43]. For imaging, larvae were treated with 0.003% phenylthiourea (PTU) to inhibit melanin pigment formation.

2.2. Angiography, calcein and DRAQ5 immersion

Microinjection was performed using a micromanipulator and a PV830 Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL) as previously described [40]. The needle was inserted directly into the heart of an anesthetized larva. Calcein fluorescence was monitored right after injection. Calcein solution (Sigma Chemicals) 0.2% wt., was prepared by dissolving 2 g of calcein powder in 1 l water and adjusting the pH to 7 using NaOH and HCl [36]. The larvae were then immersed in this solution for different time periods (5–30 min), depending on their developmental stage. After rinsing the larvae in embryo water 3 times, they were imaged using a confocal fluorescence microscope. Note that the minimum time needed to start monitoring after injection was longer than the time lapse needed for appearance of the label in the bone, i.e. when the fish was first observed the bones were already fluorescently labeled.

DRAQ5 solution (BioStatus, United Kingdom) 0.25 × 10−3 v/v, was prepared by diluting 1 μl of DRAQ5 solution in 4 ml water. The larvae were immersed in this solution for 5 h and rinsed in water before imaging.

2.3. Fluorescence imaging

An epifluorescence stereomicroscope (Leica M167FC) was used to image whole mount larvae. Pictures were taken using Leica Application Suite imaging software version 3.7 (Leica, Wetzlar, Germany). In-vivo imaging was performed on a Zeiss LSM 780 upright confocal microscope (Carl Zeiss, Jena, Germany) with a W-Plan Apochromat ×20 objective, NA 1.0. The green and red proteins and dyes were imaged sequentially as follows: green fluorescent proteins and calcein were excited at 488 nm and the emission was collected at 492/577 nm. Red fluorescent

![Fig. 1](image-url)
proteins were excited at 561 nm, and the emission was collected at 568/629 nm. The DRAQ5 was excited at 633 nm, and the emission was collected at 644/735 nm. The larvae were anesthetized with tricaine–methanesulfonate (MS 222) (0.12%) and mounted in low-melting point-agarose (1.5%). Imaging was performed as previously described [44], using a custom-built chamber for perfusion of larvae with temperature-controlled physiological medium. Z-stacks were acquired at 1.5 μm increments, every 1 min. Images were processed off-line using ImageJ (NIH) and Avizo (FEI).

2.4. Embedded samples—TEM

Zebrafish larvae were fixed in 4% paraformaldehyde in cacodylate buffer and then dehydrated in ethanol carried out in 6 steps, each twice, for 10 min: 25%, 50%, 75%, 96%, and 100%. Larvae were then embedded in Epon (EMbed 812, EMS, USA), in 6 steps, each step for 8 h: 10%, 25%, 50%, 75%, 96%, and 100% resin overnight and final embedding in a mold for 48 h at 60 °C. Ultrathin sections (70–90 nm) of the Epon embedded samples were cut by an ultramicrotome (UCT, Leica), double stained with Reynolds’ lead citrate (10 min) and 2% uranyl acetate in ETOH (50 min). The samples were then viewed and photographed with an FEI Tecnai SPIRIT (FEI, Eindhoven, NL) transmission electron microscope operated at 120 kV and equipped with an EAGLE CCD camera.

2.5. Cryo-SEM

Tails were dissected from 25 to 30 dpf zebrafish, and were immersed immediately in 10% dextran (Fluka), sandwiched between two metal discs (3 mm diameter, 0.1-mm cavities with a flat cover above), and cryoinmobilized in a high-pressure freezing device (HPM10; Bal-Tec). The frozen samples were removed from the cavity, mounted on a holder under liquid nitrogen and transferred to a freeze fracture device (BAF 60; Bal-Tec) using a vacuum cryotransfer device (VCT 100; Bal-Tec). Samples were fractured at a temperature of −120 °C, etched for 10 min at −105 °C at a vacuum better than 5 × 10 −7 mbar. This preparation procedure was used to generate a transverse view of the bones (Fig. 2). Samples were observed in an Ultra 55 SEM (“Zeiss”, Germany) by using a secondary electron in-lens detector and a backscattered electron in-lens detector (operating at 1 kV) in the frozen-hydrated state by use of a cryostage at a temperature of −120 °C.

2.6. Correlative fluorescence–Raman

The setup is based on the commercially available confocal Raman microscope (Alpha 300, WITec, Ulm, Germany) equipped with a helium neon (HeNe) laser (633 nm) laser excitation and piezoscanner (P-500, Physik Instrumente, Karlsruhe, Germany). The spectra were acquired with a thermoelectrically cooled CCD detector (DU401A-BV, Andor, UK) placed behind a grating (600 g mm −1) spectrograph (UHTS 300; WITec, Ulm, Germany) with a spectral resolution of 3 cm −1. The red laser beam (633 nm) was focused through a 10× (Nikon, NA = 0.2) and 60× water immersion (Nikon, NA = 1.0) microscope objectives. A pinhole size of 50 μm, was used. ScanCtrlSpectroscopyPlus software (version 1.38, Witec) was used for measurement and WITec Project Plus (version 2.10, Witec) for spectra processing. The implementation of the fluorescence modality relies on the substitutions of some of the original optics, integration of excitation light in addition to the white illumination light, and the addition of a module in the imaging arm, as described in [45].

2.7. Microbeam X-ray fluorescence (XRF)

In situ XRF measurements were obtained at the Nano Focus beamline ID 13 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. Samples were embedded in agarose and fixed on a kapton foil, which was clamped on a lead-tape window. The sample was mounted on a y–z scanning table and linear scans in areas of interest were performed with a step size of 1 μm using a monochromatic...
X-ray beam with an energy of 14 keV. Fluorescence spectra were collected with a fluorescence detector in transmission geometry at an angle of about 45°. The K-shell fluorescence yield of calcium, normalized to the primary beam intensity, was used to qualitatively map the position of bone segments within the tissue.

2.8. Analyzing SEM and fluorescence data

Fluorescence images were processed using ImageJ (National Institutes of Health, Bethesda, MD), and Avizo (VSG). Adobe Photoshop (San Jose, CA) software was used for adjusting brightness and contrast levels and for pseudo-coloring.

3. Results

3.1. Description of the tissue

We begin by describing the anatomy of the developing caudal fin at high resolution using TEM and cryo-SEM, followed by confocal imaging of Tg(osm:mcCherry) zebrafish, which carry a fluorescent reporter in osteoblasts. The TEM images of an embedded sample show similar features to those reported in the literature for larval zebrafish tail, including the bone hemi-cylinders, the blood vessels and the associated tissues (Fig. 2a,b). The bones are easily detected by their unique hemi-cylindrical shape and mineralized bone texture. The artery is located between the two rays. The vein, which is larger in diameter than the artery, is located between adjacent rays. Two to three layers of cells separate the artery from the bone. Some of the cells, located in close proximity to the vein, extend to the edges of the hemi-cylinders (Fig. 2a,b arrows). Confocal imaging of Tg(osm:mcCherry) larvae, which are labeled with mCherry (red) in osteoblasts (ostex), and calcein (green) in bone, revealed the location of the osteoblast cells relative to the bones (Fig. 2c). Surprisingly, the ostex-positive cells were closely detected at the edges of each hemi-cylinder (Fig. 2c, arrow), but not completely surrounding the bone surface (Fig. 2c, arrowhead).

A comparison between the fluorescence images of the ostex-positive cells and the TEM images suggests that the cells observed at the edges of the hemi-cylinder bone cross section are osteoblasts. The cell morphology in the TEM, however, does not clearly identify the osteoblast cells. To obtain a better morphological characterization of these cells, we carried out cryo-SEM on transverse sections of the rays (Fig. 2d). The sample preparation process in cryo-SEM involves only rapid high-pressure freezing, allowing preservation of the tissue under conditions that resemble its native state. In contrast, sample preparation for conventional TEM requires more invasive pre-treatment such as fixation, dehydration, staining and embedding, which can affect the shape, texture and composition of the biological specimen. The cryo-SEM images correlate well with the TEM images as far as the bone, artery and cell locations are concerned. The cell morphology and the general dimensions are, however, substantially different. These differences are attributed to dehydration process in the TEM, whereby pronounced unidirectional collapse of the soft tissue between the rigid bone hemi-cylinders occurs. As a consequence, the bone hemi-cylinders appear much closer to each other in the TEM, and the cells at the edges are deformed. Different cell morphologies are detected in cryo-SEM (Fig. 2d): the cells that are located at the edges have rounded shapes (arrows). The rounded cell location is similar to that of the ostex transcription factor expressing cells in the fluorescence images. These cells are pre-osteoblasts and/or osteoblasts. Some, but not all of the longer and thinner cells that line the bones, and are ostex-positive, are also assumed to be osteoblasts. In addition, cryo-SEM micrographs show a membrane surrounding the whole bone complex.

3.2. In vivo calcein labeling experiments

Live zebrafish larvae were immersed in water containing the fluorescent marker calcein (0.2% wt.) and were imaged using confocal microscopy. Two zebrafish transgenic reporters highlighting endothelial cells were used in these experiments: Tg(fli1:DsRed) (red fluorescence) and Tg(fli1:EGFP) (green fluorescence). Uptake of calcein from the water into the larval bones was found to take place within ~3 min for 6 dpf larvae, and 30 min for 30 dpf larvae (Fig. 1a) [36]. Immediately after immersion, the fish gut is strongly stained by the calcein dye, suggesting that the calcein passes directly through the digestive system and not by diffusion through the fish epidermal layer. Note that dead larvae do not take up calcein. When calcein (0.2% wt.) was injected directly into the blood circulation using microangiography (~25 dpf), the bones were labeled with calcein within a few minutes following the injection. Because of the quasi-instantaneous appearance of the label in the bone, it is conceivable that calcein is transported to the bone surface in part directly from the blood vessels. At later stages (~35 dpf), when the initial formation of the caudal fin is complete, bone labeling by immersion of the fish in calcein solution takes several hours, indicating that a different mechanism for calcein transport or interaction might be active.

3.3. Calcium deposits in close association with blood vessels

At ~25 dpf, in addition to the calcein stained bones, we also observed concentrated calcein stained particles, and particle aggregates (Fig. 3a, arrow and arrowhead). Some of these aggregates were detected close to the bone rays (Fig. 3a, arrowhead, c, d), but many were located at some distance from the bone rays and close to the blood vessels (Fig. 3a, arrow). A strong calcein signal was observed from some particles close to the bone within the cytoplasm of fli1-positive cells (Fig. 3c, Supplementary Movie 1, and Supplementary Fig. 1). To confirm that the fli1 signal is indeed from cells which carry mineral particles, the live nuclei stain DRAQ5 was used. We imaged Tg(fli1:EGFP) larvae, labeled with calcein (mineral particles), and DRAQ5 (far red emission). Ideally we could have demonstrated this using calcein blue to optimize the contrast between the fli1 EGFP green label and the calcein label. Unfortunately the signal from calcein blue is weak, and thus difficult to identify. Despite the problem of using two green labels, the intensity differences still enable us to visualize mineral particles located adjacent to the cell nuclei in fli1-positive cells. We therefore confirm that the calcein dense particles are intracellular (Fig. 3d, supplementary Fig. 2). Intracellular vesicular bodies of sizes <1 μm, which closely resemble mineral particles, were observed by cryo-SEM in bone-lining cells (Fig. 3e). Small calcein fluorescent particles (~1 μm) were also distributed all over the tail (Fig. 3a). In some cases, the small particles formed larger aggregates, (~7 μm) (Fig. 3a, arrow), which were more abundant in the distal part of the tail, where the bone was not yet mineralized. In all cases, the calcein stained aggregates were located very close to a blood vessel (stained red in the figures). To confirm that the calcein labeled aggregates are indeed tagging calcium mineral particles, we used X-ray fluorescence (XRF) (Fig. 3b) with 1 μm beam resolution to localize and map the calcium distribution in the entire tissue of a zebrafish tail. We observed small particles (~1 μm) and larger particle aggregates of ~7 μm that contain calcium (Fig. 3b inset) at locations where we had previously observed calcein binding aggregates. Maps of phosphorus and sulfur obtained in the same areas show particles that have co-localization of calcium, phosphate and sulfur (supplementary Fig. 3). We therefore conclude that the calcein fluorescence is mapping deposits of a calcium phosphate mineral in the tail fin tissue. The large mineral-containing aggregates were detected mainly in the regions where the bone had not yet formed, or was just forming. It is not clear whether the large aggregates are in vesicles within or outside cells or whether these aggregates are associated with the collagen matrix of the soon-to-be mineralized bone.
3.4. Calcium deposits between bone hemi-cylinders

Small particles with high calcein concentrations, presumably labeling mineral deposits, were observed between the artery and the inner bone surface (Fig. 4a, Supplementary Movie 1). Cryo-SEM images of the hemi-cylindrical bones and the associated soft tissue occasionally show clusters of vesicles (Fig. 4c, d) in locations equivalent to those of fluorescent particles. The vesicles in both images (Fig. 4a, c) are in the sub-micrometer size range, and contain mineral (see next section).

3.5. Identification of the mineral phase in the aggregates

In order to obtain information on the mineral phase in vivo, we used Raman microspectroscopy correlated with fluorescence imaging [45]. In this correlative approach, the fluorescence and Raman scattered light share the same optical path and can be detected simultaneously. We first used fluorescence imaging to localize the region of interest (ROI), and then acquired the Raman spectrum from the very same ROI. The spatial resolution of the Raman confocal microscope is limited by the depth of the acquisition of the scattered light in the tissue. Under the conditions used (see Methods), the lateral and axial resolutions are ~1 μm and 3 μm respectively.

Anesthetized Tg(fl1:EGFP)y1 nacre fish without pigments [41] were mounted on a microscope slide after immersion in calcein solution for 30 min and rinsed in water. The bones and blood vessels were identified by means of fluorescence contrast (Fig. 5a). The mineral aggregate distribution is not homogeneous throughout the tail. In order to locate regions of high mineral concentrations between two bone hemi-cylinders, we scanned the bones in the XY plane and the ZY plane (Fig. 5b). Following this procedure, mineral was located between the two hemi-cylinders, at a distance that was at least twofold larger than the resolution, thus ensuring that the detected signal did not originate from the bone itself. The bone spectrum (Fig. 5c, black) shows
characteristic features of newly deposited bone, namely the mineral PO$_4^{3-}$ vibration (labeled $\nu_1$ PO$_4$ at 960 cm$^{-1}$), and the characteristic HPO$_4^{2-}$ spectral signatures that are characteristic of freshly deposited bone mineral [46]. In contrast to this newly deposited bone, the mineral aggregates in the tissue generated spectra with phosphate $\nu_1$ PO$_4$ peaks of lower intensity (Fig. 5, blue and red spectra; supplementary Fig. 4 for the non-mineralized soft tissue reference), and spectral features (Fig. 5, inset) characterized by: (i) a shift of the main $\nu_1$ PO$_4$ peak from 960 cm$^{-1}$ to 955–958 cm$^{-1}$ (the range observed for several calcium phosphate deposits that were found next to bones); (ii) presence of a peak at 967 cm$^{-1}$; and (iii) a broad peak centered at ~945 cm$^{-1}$. The main $\nu_1$ PO$_4$ peak of the mineral deposits centered at 955–958 cm$^{-1}$ is neither a typical Raman amorphous calcium phosphate (ACP) peak, nor a typical peak of carbonated apatite. ACP with a Ca/P ratio of 1.4 is known to absorb around 950 cm$^{-1}$ [46,47], and carbonated apatite at 960 cm$^{-1}$ [46]. The $\nu_1$ PO$_4$ peak at 967 cm$^{-1}$, accompanied by the 955 cm$^{-1}$ peak, has been attributed to an octacalcium phosphate like (OCP-like) phase [46]. A similar set of peaks was observed by Crane et al. [17] using Raman spectroscopy in the coronal suture of a mice tissue culture, and was assigned to an OCP-like phase (955 cm$^{-1}$ and 970 cm$^{-1}$) and ACP (945 cm$^{-1}$). Reference spectra of ACP, OCP and carbonated HA are shown in supplementary Fig. 5 for comparison.

Interestingly, in both newly deposited bone and mineral aggregates, a series of peaks in the range of 700 to 800 cm$^{-1}$ were observed. These peaks can be assigned to the ring breathing modes of pyrimidine bases (C, T, and U) of DNA and RNA related molecules, as well as the phosphodiester bridges [48–52]. Additionally, a peak located at 1090 cm$^{-1}$, that can be assigned to the phosphodiolxy symmetric stretching (e.g. nucleic acids backbone), was observed in the mineral deposits [52] (supplementary Fig. 4).

4. Discussion

Here we show that intra-cellular and extra-cellular micrometer-sized mineral deposits are formed in tail fin tissues at some distance from the forming bones, and in many cases in proximity to the forming vascular system. Mineral deposits close to the forming fin bones were also observed, as was previously reported in adult zebrafish [12]. Our results suggest that during bone formation, the mineralization pathway in zebrafish involves other cells and processes, over and above the cells adjacent to the forming bone, which deposit the initial disordered mineral phase and transport it into the extracellular bone matrix. An analogous observation was made in the developing sea urchin larvae, where mineral deposits were observed in cells not known to be directly involved in spicule formation [53].

The intimate association of the vascular system with forming bones [54,55] is confirmed by showing that in the forming zebrafish tail, both ingested calcein, and calcein injected directly into the blood stream, reach the tail fin tissues and stain the bones within minutes. Blood circulation is clearly intimately involved in calcium transport. This in turn raises the question of whether or not calcium phosphate aggregates form in the blood itself [7,8].

The time scale for bone labeling by calcein changes dramatically when the larvae reach the age of ~35 dpf. At this stage, the initial formation of the caudal fin is complete. Prior to this stage, only a few minutes are required for the bone to be homogenously labeled. After this stage (~35 dpf), bone labeling by immersion of the fish in calcein solution takes several hours. This may indicate that different mechanisms for calcein transport are active before and after 35 dpf. Assuming that calcium and calcein share the same pathway, we infer that during the early stage (~35 dpf) when initial bone mineralization occurs, the
mechanisms for ion transport involve direct and fast pathways involving cells from endothelial cell lineage. During this time, the forming bone is relatively thin (1–2 μm) and less compact compared to adult zebrafish fin bone (~10 μm) [12]. When the initial skeleton is complete, the bones start thickening and increasing in overall size. We presume that the bone lining cells and osteoblasts are actively involved in this stage of bone formation, whereas in the larval stage they may be less active.

One type of mineral deposit that we identified is located relatively far from the mineralizing bone, but is in close proximity to blood vessels. Some of these mineral particles have sizes of several microns and do contain calcium and phosphorous. We have not identified their mineral phase, nor do we know that this mineral is ultimately deposited in the bone. Their presence at some distance from the forming bone raises the possibility that this mineral may have additional functions. We do however note that within a short time after their deposition, the distal ends of the forming bones will be close to this region, raising the possibility that these are reservoirs of mineral to be used for future bone mineralization.

Different groups of mineral deposits are found in intracellular locations, between bone rays and in close proximity to the forming bone. Surprisingly, the cells containing the mineral deposits were identified as belonging to the endothelial cells lineage based on the expression of DsRed driven by the fltl promoter. This implies that at least some of the cells that line the forming bone outer surface are endothelial in origin. The possibility that osteoblasts differentiate from endothelial cells was proposed in studies that describe the intimate relationship between the capillary system and bone in different animal models such as sheep and mouse [56–58]. It was suggested that endothelial cells and/or pericytes could serve as precursors for osteoblasts, and hence take an active part in bone formation. Furthermore, recent studies reported on coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone [59].

The (pre)osteoblasts observed in the Tg(osx:mCherry) fish, were located mainly at the edges of the forming bone semi-cylinders, and only rarely next to their flat surfaces. The location of the pre-osteoblasts at the edges of the semi-cylinders coincides with that of the fltl+ cells. Taken together, this indicates that at this stage of larval development, bone mineralization occurs mostly at the edges of the semi-cylinder surfaces. The osx+ cells that are located at the center of the semi-cylinder and line the bone surface, are more elongated than the osx+ cells at the edges, and resemble mature osteoblasts as characterized by TEM in human and rat bones [60,61]. Cryo-SEM shows intracellular clusters of vesicles in the same locations where mature osteoblasts are observed (Fig. 4c,d), and calcine-labeled particles are observed by in vivo imaging at similar locations.

**Fig. 5.** In vivo fluorescence-Raman imaging: (a) Confocal image taken in the XY plane, of one semi-cylinder bone with a blood vessel located at the center. Calcine labels the bones (Bo) and EGFP the EC and blood vessel (BV). The EC carries a homogeneous fluorescence signal in the cytoplasm of the cell, resulting in the fluorescence marker of the blood vessel wall. The high fluorescence signal in the center of the EC is contributed to the nucleus region of the EC. The micrograph was taken in vivo using the correlative fluorescence-Raman microscopy. The white circles correspond to the Raman laser beam size. Scale bar 20 μm. (b) Schematic figures of the planes scanned using the correlative fluorescence-Raman microscopy. (c) Raman spectrum of bone (black) and two spectra of mineral aggregates located between two semi-cylinders of one ray (red and blue). Inset: enlargement of the region delineated by the rectangle.
Mahamid et al. observed mineral vesicles in cells located next to the bones in the adult zebrafish tail, using cryo-SEM [12]. However, the cells were not shown to be osteoblasts. Knopf and coworkers [26] followed the formation of new bones in the mature zebrafish tail after amputation using three different osteoblast markers (runx2, osterix and osteocalcin), and observed osteoblasts located along the direction of growth of the bone rays. No information is available, however, on whether these osteoblasts were associated with mineral deposits. The data reported here are in agreement with the observations by Mahamid et al. and Knopf et al. [12,26], and provide a more comprehensive view of mineral deposition and cell activity during the initial stages of bone formation in larval zebrafish.

The mineral phase was investigated using in vivo Raman microspectroscopy coupled with fluorescence imaging, which provided spatially resolved chemical information. The spectral features observed in locations corresponding to intra-cellular mineral deposits (main PO₄ peak positions: 955–958, 967, 945 cm⁻¹), suggest that the mineral phase in the aggregates is similar to the phase that was observed by Crane et al. in the coronal sutures of embryonic mice tissue culture. Crane et al. [17] identified a complex mixture of amorphous calcium phosphate and disordered OCP-like mineral phases. No evidence for a polyphosphate phase (reported in murine bone forming cells [16]), which is expected to have peaks at 700 and 1180 cm⁻¹ [62], was observed. The mineral aggregates that we observed between bone hemi-cylinders are presumably intra-cellular, and represent the earliest stage of mineralization. As such, we do not expect them to be a crystalline phase, but rather a disordered calcium phosphate phase complex, which may have some characteristics of an octacalcium phosphate mineral.

The Raman spectra also reveal the presence of nucleic acid-like molecules in close proximity to the mineral. Due to the limited axial resolution, we were not able to determine whether these nucleic acid-like molecules are directly associated with the mineral phase or if they appear in the spectra as a result of averaging the signal of the mineral aggregate with that coming from the surrounding tissue. We note that ATP has been reported to be associated directly with ACP in the crab hepatopancreas [63] and that in vitro studies showed that ATP delays the conversion of ACP to crystalline carbonated apatite [64]. The presence of DNA-like species in association with the mineral phases introduces an interesting perspective on the possible role of such species in stabilizing transient disordered phases on their way to the mineralized tissue.

5. Conclusions

We demonstrate the presence of mineral aggregates in close proximity to the blood vessels, between and within the intra-ray region, and relatively far from the bone. The aggregates consist of a disordered calcium phosphate phase with characteristic features of OCP. The observation that mineral particles form at diverse locations, and involve different cell types including cells which express fltl fluorescent reporter entails a conceptual change in our understanding of the comprehensive mechanism of bone formation in vertebrates, from the blood to the bone.

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References


