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Fourier Transform Infrared Spectroscopy of Developing Bone Mineral: From Amorphous Precursor to Mature Crystal

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Abstract

Bone mineral development has been described to proceed through an amorphous precursor prior to apatite crystallization. However, further analytical approaches are necessary to identify specific markers of amorphous mineral components in bone. Here, we establish an original Fourier transform infrared (FTIR) spectroscopy approach to allow the specific identification of the amorphous and/or crystalline nature of bone mineral. Using a series of standards, our results demonstrate that obtaining the second derivative of the FTIR spectra could reveal a peak specifically corresponding to amorphous calcium phosphate (ACP) at $\sim 992\text{ cm}^{-1}$. The intensity of this peak was strongly correlated to ACP content in standard mixtures. The analysis of a variety of bones showed that a clear ACP peak could be identified as a specific marker of the existence of an amorphous mineral component in developing bones. In contrast, the ACP peak was not detected in the mature bones. Moreover, subjecting developing bones to *ex vivo* crystallization conditions led to a clear reduction of the ACP peak, further substantiating the conversion of amorphous mineral precursor into mature apatite crystals. Analysis of mineralization in osteogenic cell cultures corroborated our observations, showing the presence of ACP as a major transient component in early mineralization, but not in the mature matrix. Additionally, FTIR imaging revealed that ACP was present in areas of matrix development, distributed around the edges of mineralizing nodules. Using an original analytical approach, this work provides strong evidence to support that bone mineral development is initiated by an amorphous precursor prior to apatite crystallization.

INTRODUCTION

The ability to form biologically driven mineral is widespread in many organisms across all evolutionary kingdoms, resulting in a rich variety of types of mineral and mineralized structures, each intrinsically designed with its own particularities and functions^{1–4}. The understanding of mechanisms underlying the biomineralization process is of great

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importance not only from a fundamentally scientific point of view, but also as source of inspiration for the development of novel materials, technologies and therapeutic approaches to improve quality of life ^{5–7}. A key question in biomineralization is how the process is initiated. The existence of amorphous mineral precursors prior to mineral crystallization has been proposed to be a widespread phenomenon in biomineralization ^{8–11}. Many different organisms have been observed to begin biomineralization by first forming a structurally disordered mineral phase, which gradually transforms into mature crystals during development. It is thought that this strategy allows crystals to be more easily molded by the biological environment and molecular interactions, as well as infiltrate the organic matrix, achieving properties that favor their biological function ^{12–14}.

The pathway of mineral crystallization may involve different stages, in which atoms progressively aggregate and rearrange, forming amorphous particles that act as nuclei for the development of crystalline domains ^{15–17}. This process is marked by an increase in the degree of order of the atomic structure, which can be characterized across hierarchical length scales. Amorphous materials are ordered only at the scale of chemical bonds and atomic interactions in the local molecular environments (short-range order), whereas crystalline materials display a periodical arrangement of atomic structures repeated throughout the crystal lattice dimensions (long-range order) ¹⁸. To identify the amorphous and crystalline nature of the mineral, specific features of the short-range and long-range order can be assessed by using appropriate analytical approaches sensitive to structural order at different length-scales. For instance, long-range order analysis are unveiled by methods such as X-ray diffraction (XRD), electron diffraction and high-resolution transmission electron microscopy, which assess the periodicity and spacing of atomic planes that repeat along the crystal directions ¹⁹. In contrast, methods such as Fourier transform infrared (FTIR) and Raman spectroscopy can provide direct information about the short-range order of materials, being sensitive to characteristics of molecular vibrations arising from chemical bonds between atoms in the material ²⁰. In this context, it is essential to apply the appropriate analytical approach to investigate specific markers of amorphous and crystalline materials.

Since the late 1960s, transient amorphous precursors have been widely described in the biomineralization of invertebrates, such as: ferrihydrite (disordered iron hydroxide) ²¹ and amorphous calcium phosphate (ACP) ²² in the radular teeth of chitons (mollusk) and amorphous calcium carbonate (ACC) in spines of sea urchins (echinoderm) ²³, dorsal carapace of blue crabs (crustacean) ²⁴ and calciferous gland of earthworms (annelid) ²⁵. Several studies described that the mineral found in vertebrate bones may also be initially assembled as an amorphous component, which acts as precursor for the poorly crystalline apatite crystals found in mature bone. The presence of ACP in bones was first suggested over 50 years ago ^{26, 27}. With the availability of newer analytical approaches, stronger indications of an ACP component in vertebrates have been shown in mouse teeth enamel ²⁸, zebrafish fin bones ^{29–33}, mouse calvaria and long bones ³⁴, and chicken femurs ³⁵, as well as in osteogenic cell culture systems ³⁶. It is important to mention that the presence of ACP as a precursor to apatite development has long been and remains a topic of controversy and dispute in the bone biomineralization field ^{37–43}.

In previous studies^{29, 30, 34, 35}, developing bones were analyzed chiefly from a structural point of view, presenting beautifully detailed images of the tissue nanostructure obtained by electron microscopy and microanalytical approaches. They show by electron and XRD analysis that some particles had an absence of crystalline pattern, indicative of the presence of ACP in bones. Some studies have used spectroscopic approaches to evaluate the short-range order of bone mineral^{29, 31–33, 35, 44}, but the identification of specific ACP markers in bone were not conclusive. Although these previous studies show significant advances in understanding bone biomineralization, it remains a key task to apply different analytical approaches in the search for specific markers that support the presence of ACP in developing bone.

Here, we used an FTIR spectroscopic approach to specifically identify the amorphous and crystalline nature of the mineral present in a variety of bones, as well as in osteogenic cell cultures. FTIR is a well-established and very powerful technique of vibrational spectroscopy, which allows the specific identification of substances based on their intrinsic chemical and molecular structure, composition and local environments^{45, 46}. In particular, the peaks in the FTIR spectra arise from vibrations of specific atomic bonds, which absorb infrared light and give rise to characteristic bands in precise position (frequencies) of the spectrum. These bond vibrations can be different modes, such as symmetric and asymmetric stretching (ν_1 and ν_3) and bending (ν_2 and ν_4), yielding specific bands. Moreover, it is possible to use mathematically objective approaches (such as obtaining the second derivative of the spectra) to enhance peak resolution by producing sharper peaks with very precise positions, improving the identification of substances with greater specificity^{47, 48}. Therefore, we hypothesized that FTIR can be used for the identification of a specific ACP marker in bone.

This work provides direct evidence for the existence of ACP in developing bones, an amorphous phase which was not observed after natural bone maturation or after *ex vivo* crystallization. Our results support that bone mineralization proceeds through a transient ACP precursor prior to apatite crystal development, highlighting the role of amorphous mineral precursors in the biomineralization of vertebrates. Although previous studies have described ACP as a mineral precursor in bone formation^{29–36, 44}, a contribution of our findings is in the use of an analytical approach that allowed the identification of ACP based on a clear, specific spectral marker. Moreover, using a larger variety of samples, a minimal preparation procedure and *ex vivo* crystallization assays, our findings provide a compelling argument that significantly adds to previous studies on the existence and role of ACP in bone.

MATERIALS AND METHODS

Synthetic standards

Standards were obtained using a well-established method for preparation and conversion of synthetic ACP into apatite⁴⁹. Briefly, 0.04 M calcium chloride and 0.036 M dibasic phosphate solutions were prepared in 0.15 M Tris-HCl buffer pH 8.5. The solutions were mixed in a 3:4 ratio, leading to the immediate precipitation of ACP, which was spun down, washed with ethanol and lyophilized overnight. Aliquots of the dry sample (time 0) were immersed in Tris-HCl for either 1h or 24h at room temperature to allow progressive

crystallization into apatite, followed by subsequent washes and lyophilization. The ACP/apatite mixtures were prepared by mixing known amounts of samples obtained at time 0 (ACP) and at 24h crystallization (apatite). All samples were analyzed in triplicate.

Bone samples

Immature bones at various stages of development and mature bones were collected from Sprague Dawley rats, C57Bl6/J mice and zebrafish (*Danio rerio*). Immature bone samples were obtained from the calvaria and long limbs (femur and tibia) of rat fetuses collected at two different embryonic ages (E18 and E21), newborn mice (day 0–1), and the caudal fin bone of adult zebrafish (1.5 years old), which are known to continually develop and thus contain regions of immature mineral^{29, 30}. Mature bone samples were obtained from the calvaria and long limbs of adult rats and mice and from the skull of the zebrafish. All samples were frozen immediately after collection and stored at –80 °C until analysis. Prior to analysis, each sample was thawed individually, and the bones of interest were dissected to remove adjacent tissues, dehydrated in absolute ethanol for a few minutes, quickly frozen by immersion in liquid nitrogen, ground into a powder (around 100 µm particle size) and immediately analyzed by FTIR spectroscopy. The whole process from dissection to spectra collection was performed within 15 minutes. At least five samples were analyzed from each animal and age group. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Temple University.

Ex vivo crystallization

Powdered samples of developing bones that contained immature mineral (calvaria of rats at E18 and the distal tips of zebrafish caudal fin bones) were exposed to solution that allowed crystallization of ACP into apatite. The samples were immersed in 0.15 M Tris-HCl buffer pH 8.5 for 24h to favor the *ex vivo* crystallization of ACP into apatite, spun down, washed with ethanol and lyophilized overnight. The assay was repeated with at least three samples of each bone type.

Cell culture

The cells used in this study were 7F2 murine osteoblasts, with hFOB 1.19 human osteoblasts used for additional experiments (American Type Culture Collection). *In vitro* bone mineralization assays were performed by culturing cells in micromasses under osteogenic conditions for 1 and 2 weeks. Briefly, cells were seeded onto infrared-transmissive silicon wafers in 5µL drops of medium containing 50,000 cells each and kept still for 90 min to favor cell attachment and formation of micromasses. The cells were then cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 50 µg/mL ascorbic acid and 2 mM β-glycerophosphate at 37 °C in a humidified 5 % CO₂ incubator. After 1 or 2 weeks, samples were dehydrated in ethanol for 10 minutes and either imaged by FTIR imaging or scraped off and powdered for FTIR spectroscopy. The assay was repeated three times, with at least three micromasses per timepoint.

X-ray diffraction

Samples were analyzed in a Bruker d8 Advance Powder X-Ray diffractometer, with data collection parameters of 0.02° 2θ step size and 300 sec/step. The diffraction peaks were analyzed using OriginPro software (OriginLab) and indexed following the Powder Diffraction File database PDF-2 (International Centre for Diffraction Data), specifically the standard file of hydroxyapatite (#9–432).

Fourier transform infrared spectroscopy

Samples were analyzed by FTIR using the attenuated total reflection (ATR) sampling technique in a Thermo Scientific Nicolet iS5 FTIR spectrometer equipped with an iD7 ATR accessory with a diamond crystal. The powdered samples were placed on the crystal and spectra were collected with 4 cm^{-1} resolution and 32 co-added scans. The degree of mineralization of the bones was assessed by the integrated band area ratio of the $\nu_3\text{ PO}_4$ (mineral) to the amide I (protein) absorbances using OriginPro software⁵⁰. The second derivative of the spectra were obtained using The Unscrambler X software (CAMO), applying the Savitzky-Golay algorithm with 7 smoothing points to achieve a good compromise between signal-to-noise and spectral features. As second derivative peaks are negative, the spectra were inverted for an easier visualization. The second derivative spectra were utilized to resolve broad absorbances and evaluate specific peaks associated with apatite and ACP.

Fourier transform infrared spectral imaging

Cell cultures of 7F2 osteoblasts were analyzed by FTIR imaging in a PerkinElmer Spotlight 400 spectrometer, with $6.25\text{ }\mu\text{m}$ spatial resolution, 4 cm^{-1} spectral resolution and 8 co-added scans. Intact micromasses on Si wafers were imaged in transmittance mode and analyzed using ISys software (Malvern Instruments), to obtain the distribution images of the mineral and protein components based on the area of the $\nu_3\text{ PO}_4$ and amide I bands, respectively. The spatial correlation analysis between mineral and protein distribution was done using ImageJ software (National Institutes of Health). Briefly, pairs of grayscale images were analyzed with the “colocalization threshold” plugin, to quantify the pixel intensity spatial correlations of components from both images. The Pearson correlation coefficient (R) and percent areas of correlation were obtained as the quantitative outcomes of the analysis. To build overlay images of apatite and ACP, grayscale images were obtained based on the positive values of the inverted second derivative peaks at $\sim 1015\text{ cm}^{-1}$ and $\sim 992\text{ cm}^{-1}$, peaks unique to apatite and ACP, respectively, using ISys software. Corresponding images were set to red and green channels and merged using ImageJ software.

Raman spectroscopy

Raman spectroscopy was used to further assess direct evidences of ACP in developing bone and strengthen the observations made using FTIR. Samples were analyzed with a Horiba Labram HR Evolution spectrometer, coupled to an Olympus BXFM-ILHS optical microscope. Spectra were collected using the 532-nm excitation laser and a 100x objective lens, with 16 co-added scans of 16-sec acquisition time each and gratings of 600 lines/mm.

The laser beam spot size was 1 μm , the power at the sample surface was ~ 15.76 mW, and the spectral resolution was 5.57 cm^{-1} . The spectra were analyzed using OriginPro software.

Alizarin red staining

The 7F2 osteoblasts cultures were also analyzed by histological staining for calcium deposits. Briefly, they were stained with 2% alizarin red solution for 10 min, washed thoroughly with distilled water, dried in ambient conditions and imaged with an Olympus BX53 optical microscope. The presence of mineralization was determined by strong red staining.

Statistics

All the described analyses were repeated with multiple samples (as detailed in the methods) to ensure the reproducibility of the results. The Y-axis of graphs were often adjusted to arbitrary units by 0–1 or area normalization, to facilitate data overlay and comparison of spectral contours and peak relative intensity. Statistical analysis of parameters was done with the t-test, using InStat software (GraphPad), with differences considered significant at $P < 0.05$. Correlations among parameters were assessed using the Pearson correlation (R).

RESULTS

Spectroscopic approach for the identification of a specific ACP marker

We first analyzed synthetic standards to identify specific markers of the crystallization process of ACP into apatite. XRD was used to confirm the amorphous nature of the freshly prepared standard, and to monitor changes in the long-range order of samples submitted to progressive crystallization periods. As expected, ACP at time 0 did not show any distinct features in the pattern, only a broad and nonspecific diffraction halo³⁹ (Fig. 1a). After 1 hour, the sample began to show signs of crystallization, as recognized by the appearance of small diffraction peaks from apatite lattice planes (Fig. 1a). After 24 hours, the sample presented a typical pattern of a poorly crystalline, bone-like apatite (Fig. 1a). FTIR spectral analysis of the same materials showed that all — including freshly prepared ACP — had a characteristic short-range order, as seen by absorbance bands associated with PO_4 bond vibrations (Fig. 1b). In particular, the spectrum of ACP presented clearly broader bands, with no splitting in the $\nu_4 \text{PO}_4$ region (Fig. 1b). This illustrates that XRD primarily shows specific peaks related to crystalline phases, whereas FTIR shows absorbances of both amorphous and crystalline components.

We further obtained the second derivative of the spectra to achieve a more detailed analysis of subtle and overlapping peaks. Interestingly, this type of analysis of the $\nu_3 \text{PO}_4$ absorbance band allowed monitoring of very clear changes in specific peaks, and the ability to distinguish the transformation of ACP into apatite. ACP can be directly identified by a peak at $\sim 992\text{ cm}^{-1}$, which is the main peak observed in the freshly prepared ACP at time 0 (Fig. 1c). The crystallization of ACP into apatite over time was characterized by the extinction of the $\sim 992\text{ cm}^{-1}$ peak and the appearance of the typical apatite peak at $\sim 1015\text{ cm}^{-1}$ (Fig. 1c). These results demonstrate the 992 cm^{-1} peak as a specific marker associated with ACP,

which can be a valuable original approach to search for direct evidence of an amorphous precursor in bone biomineralization.

Additionally, the FTIR spectra of physical mixtures of ACP and apatite showed that with a decrease in the amount of ACP, the spectral bands became narrower and there was a progressive split of the ν_4 PO₄ band into two distinct peaks (Fig. 2a). As the splitting of these peaks is a standard method for assessment of apatite crystallinity⁵¹, this illustrates the overall increase in the degree of crystallinity of the mixtures. Furthermore, the decrease in ACP content was characterized by the reduction of the ~ 992 cm⁻¹ peak in the second derivative of the ν_3 PO₄ band (Fig. 2b), and that the measured intensity of the peak at ~ 992 cm⁻¹ shows a strong correlation ($R=0.99$) with the relative ACP content of the mixtures (Fig 2c). This confirms that the peak at ~ 992 cm⁻¹ corresponds to the presence of ACP.

Evidence of ACP as a transient component in developing bones

To directly identify the presence of ACP in native bone, a variety of developing and mature bones of different species and types were analyzed using FTIR. Spectra of developing (Fig. 3a) and mature (Fig. 3b) bones showed typical mineral PO₄ bands and protein amide absorbances. Evaluation of the mineral-to-protein ratio showed that the degree of matrix mineralization was lower in all developing bones when compared to mature bones (Fig. 3c), illustrating the bone development progression. Interestingly, analysis of second derivatives of the ν_3 PO₄ band showed the specific marker of an ACP component in all developing bones, as seen by the distinct ACP peak at ~ 992 cm⁻¹ (Fig. 3d). In contrast, this peak was not clear in the mature bones, where it appears at most as a faint shoulder of the single apatite peak at ~ 1015 cm⁻¹ (Fig. 3e). This evident transition in the nature of bone mineral during development is illustrated by the overlay of the FTIR spectra of rat fetus calvaria at progressive developmental stages, showing the loss of the ACP component. (Suppl. Fig. S1). These results show a specific marker of ACP as a transient component of bone mineral, present primarily in developing bones and not detected in mature bones.

To strengthen our results and the validity of our approach, we also used Raman spectroscopy to obtain further evidence of the ACP and apatite spectral differences, and the short-range order of the developing and mature bone mineral, using the zebrafish bones as an example. We confirmed that whereas the mature bones showed a main ν_1 PO₄ Raman band typical of apatite at ~ 960 cm⁻¹, the spectra of the developing bones were clearly similar to the Raman spectra of the ACP standard, showing a peak at ~ 950 cm⁻¹ (Suppl. Fig. S2).

To further elucidate the role of ACP as a potential bone apatite precursor, we subjected samples of developing bones to *ex vivo* crystallization conditions that favor the transformation of ACP into apatite. Assays using developing zebrafish caudal fin tips clearly demonstrated a marked reduction of the peak at ~ 992 cm⁻¹ in the second derivative of the ν_3 PO₄ band in the FTIR spectra (Fig. 4), indicating a transition of the ACP component. Additionally, we verified this process by analysis of calvaria from rat fetuses, which showed once more the reduction of the ~ 992 cm⁻¹ peak when subjected to *ex vivo* crystallization (Suppl. Fig. S3). Together, these results support the conversion of the biological ACP component found in developing bone into mature apatite, and may illustrate the process seen in native bones.

ACP is a major transient component of early mineralization in cell cultures

We also analyzed the mineral formed in osteogenic cell cultures at progressive stages of matrix development. The mineralization of the matrix over time can be visualized by the alizarin red stained calcium deposits (Fig 5a) and by the FTIR spectra, which shows the increase of the PO_4 bands relative to the amide I band of proteins (Fig 5b). FTIR spectral images demonstrate the development of the mineralizing nodules from early (Fig 5c) to more mature mineralization stages (Fig 5d). Moreover, the mineral distribution was closely associated with the distribution of proteins in both cases, presenting a highly significant spatial correlation ($R>0.9$, $P<0.01$) and indicating that mineral deposition was biologically mediated by the cells and matrix.

Further analysis of the cultures by FTIR showed that the characteristics of mineral formed during early mineralization were different from the mineral present in the more mature matrix: the early PO_4 bands were similar to those described for ACP, whereas the mature bands were typical of bone apatite (Fig. 6a). The second derivative of the $\nu_3 \text{PO}_4$ band confirmed this observation, showing direct evidence of the specific ACP marker in the developing matrix, as seen by the prominent peak at $\sim 992 \text{ cm}^{-1}$, whereas the more mature matrix showed primarily apatite peaks, including the intense peak at $\sim 1015 \text{ cm}^{-1}$ (Fig. 6b). To strengthen this observation, we also analyzed bone mineralization in cultures of a different osteoblast cell line, which showed again the pronounced presence of ACP in the developing matrix preceding the formation of apatite in the more mature matrix (Suppl. Fig. S4). This further corroborates the observations from native bones and demonstrate the existence of ACP as a major component of the developing matrix, transiently found in early mineralization stages.

Furthermore, the mineralization nodules were investigated at micron resolution using FTIR imaging. The overall distribution of mineral in mature cultures was obtained based on the area of the $\nu_3 \text{PO}_4$ band, illustrating the mineralizing nodules (Fig. 7a). The spectra forming the whole image were averaged, and the second derivative of the $\nu_3 \text{PO}_4$ band showed typical apatite peaks (Fig. 7b), confirming the results described above with powdered samples. However, we noticed that some areas also showed the presence of ACP, as seen by the clear peak at $\sim 992 \text{ cm}^{-1}$ (Fig. 7b). Using the second derivative peaks, we obtained more specific images illustrating the distribution of apatite (Fig. 7c) and ACP (Fig. 7d) in the mineralizing nodules. Interestingly, the overlay of these images (Fig. 7e) indicates that whereas apatite is seen in the more developed core of the nodules, minor ACP components were found primarily around the edges, where the matrix is in active formation as the nodules expand. These results strengthen our observations, indicating in a spatially resolved manner the presence of ACP in areas of matrix development, but not in more mature regions.

DISCUSSION

The development and mineralization of bones is a crucial step in the life of vertebrates. To enable a better understanding of this process, we used an approach based on a spectroscopic marker to specifically identify the amorphous and crystalline nature of the mineral present in a variety of bones, as well as in osteogenic cell cultures. Our findings support the concept that the mechanism of bone mineral crystallization may involve progressive stages, which

have been illustrated in previous studies^{15, 42, 52} and are shown here by the direct identification of the specific marker of ACP and/or apatite corresponding to different stages of bone mineral development (Fig. 8). In this possible scenario, amorphous particles would act as mineral precursors into which apatite is initially nucleated, forming a crystalline core within the ACP particle. As the mineral develops, atoms from the ACP shell would be incorporated into the crystalline core, resulting in the increase of the apatite crystals and decrease of the amorphous surface layer. This process has been described during apatite maturation, with the surface layer acting as a dynamic interface where loosely bound ions can be stored, reversibly exchanged with the surrounding environment or ultimately incorporated into the growing crystalline core^{53–55}. Alternatively, it should be considered that the transformation of ACP into apatite may involve different stages and mechanisms, including the dissolution of amorphous components and reprecipitation of the crystalline phase⁴³.

In this work, our findings show direct evidence that bone mineral in developing bones comprises both ACP and apatite, possibly indicating the stage of apatite nucleation within amorphous precursor particles. In the mature bones, only apatite peaks are detected, showing typical signs of a more developed crystal with little indication of an amorphous component. Previous studies applying solid-state nuclear magnetic resonance (NMR) approaches have described that bone mineral is formed by an ACP surface layer coating the crystalline core of bone apatite^{42, 52, 56}. Here, the presence of ACP in mature bones was not detected by FTIR analysis. This was perhaps due to a limitation in our approach, which impaired the detection of very fine ACP domains in a primarily crystalline phase. In this case, the apatite peaks would overwhelm the spectra, concealing peaks from the ACP layer. However, in developing bones, in which the mineral would be comprised of more pronounced ACP domains relative to a less developed crystalline core, we show that clear peaks can be identified as specific markers of both ACP and apatite.

Besides identifying a specific marker of ACP in the different developing bones, we also performed an *ex vivo* crystallization assays with these samples, highlighting the conversion of biological amorphous precursors into crystals. The *ex vivo* crystallization of mineral from developing zebrafish bone has also been described in a previous study²⁹. The authors found that the isolated mineral particles presented an amorphous electron diffraction pattern, but after a few weeks left on the transmission electron microscopy grid, they spontaneously crystallize and produced faint diffraction spots in the pattern. Here, we present a contribution to the current *ex vivo* crystallization data by subjecting the amorphous bone samples to a buffer that actively promotes the conversion of ACP into apatite, thus supporting that the amorphous mineral found in developing bones could act as an apatite precursor. Furthermore, in the previous study²⁹, the mineral particles were isolated using a sodium hypochlorite treatment, which can lead to changes in the mineral⁴¹. Here, all bones were dissected and analyzed within minutes, preparing the samples simply by quick dehydration in ethanol and flash freezing in liquid nitrogen before grinding into a powder. Moreover, using the ATR sampling technique of FTIR allowed performing a quick and straightforward data collection immediately after sample preparation.

FTIR approaches have been used previously for the identification of an amorphous precursor in bone ^{29, 35}. Although these papers present valuable contributions to the field, the FTIR analysis was limited to the raw spectra, primarily showing broad bands from poorly crystalline apatite, with little indications of an ACP component. Considering that the FTIR bands often arise from different compositional peaks, they may need to be deconvoluted to reveal underlying and overlapping peaks and allow the identification of individual components with greater specificity ^{47, 48}. In particular, obtaining the second derivative of the spectra — the approach we used here — is strongly recommended as a mathematically objective technique to enhance peak resolution by producing sharper peaks with precise positions ⁴⁸.

It is important to also highlight previous studies that used Raman microspectroscopy to evaluate early stages of bone formation in the zebrafish larvae ^{31–33}. These papers represented a step forward on understanding the chemical nature of the mineral precursor involved in bone biomineralization. They show that HPO_4 is a major component of this precursor, suggesting that the early bone mineral is an acidic form of calcium phosphate, somewhat related to ACP, octacalcium phosphate (OCP) and/or brushite (dicalcium hydrogen phosphate dehydrate, DCPD). These results are in line with a previous study that used Raman to evaluate bone formation on the coronal suture of mice fetuses ⁴⁴. However, in their Raman spectra, a potential peak of ACP appears only as a faint shoulder on the main PO_4 band, making it challenging to reach a precise conclusion. In contrast, here we show a very intense and clear peak of ACP in the Raman spectra of the zebrafish bone, which was objectively identified by comparison to a synthetic ACP standard. Moreover, choosing to evaluate the samples by FTIR in addition to Raman allowed a straightforward identification of both ACP and apatite as components of not only the zebrafish bones, but also a variety of developing bones and cell culture samples, emphasizing the relevance of ACP as a bone mineral precursor across different species.

It is interesting to briefly discuss some differences in the analysis by FTIR, Raman and XRD of the same sample, using the zebrafish developing bones as example. The second derivative of the main mineral band of the FTIR spectra showed peaks of both apatite and ACP (Suppl. Fig. S5a). However, the main mineral band in Raman was typical of ACP, with its second derivative showing only a single ACP peak, and no identification of apatite (Suppl. Fig. S5b). The presence of a very poorly crystalline apatite, in contrast, can be identified in the XRD pattern (Suppl. Fig. S5c). This illustrates how these different analytical approaches are sensitive to distinct components of the mineral. XRD yielded information about the crystalline mineral, while Raman showed only the amorphous component — which may have overwhelmed a peak from apatite crystals in the sample. Interestingly, FTIR analysis was able to assess features of both crystalline and amorphous components, allowing identification of the presence of both apatite and ACP in the variety of developing bones described in this work.

It is important to briefly discuss the novelty of this work. In the last decade, several studies suggested that the mineral found in vertebrate bones is initially assembled as ACP, which acts as precursor for the poorly crystalline apatite crystals ^{29–35, 44}. However, the current knowledge of the existence of amorphous mineral precursors in bone relies primarily on

observations of a lack of crystallinity by electron diffraction and high-resolution imaging and on the identification of faint peaks with challenging assignments by spectroscopic methods. Although these studies contributed greatly to the understanding of bone mineral development, the search for more direct evidences based on the specific identification of ACP markers was still necessary, and hence the goal of the present work. Here, we addressed this limitation by using an analytical approach that allowed to identify specific markers related to the short-range order that characterize ACP. With an original FTIR spectroscopic approach, we were able to provide clear evidence for the existence of ACP in a variety of developing bones, contributing to the identification of an amorphous mineral precursor in the biomineralization of vertebrates.

Previous studies have used FTIR spectroscopy to evaluate the conversion of synthetic ACP into apatite^{57–59}. In these studies, differences in the raw spectra of ACP and apatite were well characterized. They described for instance, the splitting of the broad ν_4 PO₄ band of ACP into two distinct peaks as apatite crystallizes, as well as an overall increase in definition of peaks and shoulders in the ν_3 PO₄ band, as we also observed here in our data. However, these studies focused on changes in the spectra of apatite during maturation, and did not further explore the spectra of early ACP deposits in biological mineralization to show specific peaks associated with the amorphous mineral. Identifying the clear peak at ~ 992 cm⁻¹ in the second derivative of the ν_3 PO₄ band of ACP allowed us to apply an original approach and make an important contribution to the biomineralization field, showing direct evidences of a transient amorphous component found in developing bones — but not in mature.

It is possible that the ACP peak we describe here may be related to observations made previously with analysis of synthetic apatites. Gadaleta *et al.*⁵⁸ noticed a very small peak at 985 cm⁻¹ in the second derivative of the ν_3 PO₄ band of poorly crystalline apatite, which evolved into peaks at 982 and 999 cm⁻¹ during the maturation process. However, these minor peaks were not conclusively assigned by the authors. Ou-yang *et al.*⁵⁹ used a 2-D FTIR spectroscopy approach to monitor correlations between spectral features changing during ACP to apatite conversion. They used the raw spectra in their 2-D analysis, which limited their interpretation to broad aspects of the spectra. Nevertheless, the authors described several cross-correlations between regions of the spectra of maturing apatites and suggested that the spectral region at ~ 988 cm⁻¹ was related to PO₄ in non-apatitic or nonstoichiometric environments.

In conclusion, this work shows the identification of a specific spectral marker of ACP as a transient component of developing bones, supporting its key role as potential apatite precursor in the pathway of bone mineral development. We found that obtaining the second derivative of the FTIR spectra to deconvolute mineral bands could reveal a clear peak specifically correspondent to ACP. Using this original approach, combined to careful sample preparation, we were able to directly identify the existence of an ACP component in a variety of developing bones, as well as its prominent presence in early mineralization stages of osteogenic cell cultures. Moreover, we describe that this ACP component of native bones may act as a precursor for apatite crystallization *ex vivo*, illustrating how this process may occur during bone development. An important contribution of this work to previous research

in the field ^{29–36, 44} is the clear identification of ACP in bone based on a strong specific marker, supporting that the developing bone mineral is comprised by an amorphous component prior to apatite crystallization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: The presence of amorphous calcium phosphate (ACP) can be directly identified by Fourier transform infrared (FTIR) spectroscopy.

ACP was prepared (time 0) and subjected to 1h and 24h periods in a buffer that favors the crystallization of ACP into apatite. **(a)** X-ray diffraction (XRD) patterns. **(b)** FTIR spectra. **(c)** Second derivative (inverted) of the $\nu_3\text{PO}_4$ band of the FTIR spectra. While the XRD pattern of ACP shows a broad and nonspecific halo, FTIR analysis shows that the peak at $\sim 992\text{ cm}^{-1}$ observed in the second derivative of the spectra can be used as a specific marker for the direct identification of ACP.

Figure 2: The intensity of the peak at $\sim 992\text{ cm}^{-1}$ is strongly correlated to the amorphous calcium phosphate (ACP) content.

A series of ACP/apatite mixtures were prepared and analyzed to monitor changes in the peak at $\sim 992\text{ cm}^{-1}$ associated with the ACP content. **(a)** Fourier transform infrared (FTIR) spectra. **(b)** Second derivative (inverted) of the $\nu_3\text{PO}_4$ band of the FTIR spectra. **(c)** Scatter plot showing a very high correlation ($R^2 > 0.9$, $P < 0.01$) between the height of the peak at $\sim 992\text{ cm}^{-1}$ and the ACP content of the mixtures, showing that this peak is a specific marker associated to the ACP component of the samples.

Figure 3: Amorphous calcium phosphate (ACP) is evident as a component of developing bones, but not mature bones.

A variety of developing and mature bones, obtained from different species and ossification types, were analyzed using Fourier transform infrared (FTIR) spectroscopy. Developing bones were obtained from calvaria and long limbs of day 21 rat fetuses and newborn mice and from zebrafish caudal fin. Mature bones were from calvaria and long limbs of adult rats and mice and from zebrafish head. (a) FTIR spectra of developing bones. (b) FTIR spectra of mature bones. (c) Quantification of the degree of mineralization of developing and mature bones (* $P < 0.01$). (d) Second derivative (inverted) of the $\nu_3 \text{PO}_4$ band of the FTIR spectra of developing bones. (e) Second derivative (inverted) of the $\nu_3 \text{PO}_4$ band of the FTIR spectra of mature bones. It is clear that all developing bones present the specific ACP component peak at $\sim 992 \text{ cm}^{-1}$, which is mostly lost in the mature bones.

Figure 4: The amorphous calcium phosphate (ACP) component of developing bone may be an apatite precursor when subjected to *ex vivo* crystallization conditions.

The tips of the developing zebrafish caudal fin bones were analyzed and subjected to 24h in a buffer that favors the crystallization of ACP into apatite. Second derivative (inverted) of the $\nu_3\text{PO}_4$ band of the Fourier transform infrared (FTIR) spectra shows the clear reduction of the specific ACP component peak at $\sim 992\text{ cm}^{-1}$ after *ex vivo* crystallization.

Figure 5: A progressive matrix mineralization is seen in cell culture with time.

7F2 osteoblasts were seeded as micromasses and cultured in osteogenic medium for 1 and 2 weeks. **(a)** Alizarin red staining for calcium deposits. **(b)** Fourier transform infrared (FTIR) spectra. The distribution of the mineral and protein components of the cultures was obtained by FTIR imaging analysis, using the $\nu_3\text{PO}_4$ and amide I bands, respectively. **(c)** Culture at early mineralization stages (week 1). **(d)** Culture at more mature mineralization stages (week 2). The development of the matrix is shown by the clear increase in the intensity of the staining and of the PO_4 bands from mineral relative to the amide I band from protein. Moreover, spatial correlation analysis of the FTIR images showed that mineral formation was significantly associated with the distribution of proteins ($R^2 > 0.9$, $P < 0.01$).

Figure 6: Amorphous calcium phosphate (ACP) is a major transient component of early mineralization in cell culture, preceding the formation of apatite in the mature matrix. 7F2 osteoblasts were seeded as micromasses and cultured in osteogenic medium for 1 and 2 weeks. **(a)** Fourier transform infrared (FTIR) spectra. **(b)** Second derivative (inverted) of the $\nu_3\text{PO}_4$ band of the FTIR spectra. Early mineralization shows raw PO_4 bands typical of ACP, as well as the prominent ACP peak at $\sim 992\text{ cm}^{-1}$, whereas mature mineralization shows primarily apatite features, with a clear reduction of the specific ACP component peak.

Figure 7: Amorphous calcium phosphate (ACP) is present in areas of matrix development. 7F2 osteoblasts were seeded as micromasses and cultured in osteogenic medium for 2 weeks. The matrix was investigated at $6.25\mu\text{m}$ spatial resolution by Fourier transform infrared (FTIR) imaging. **(a)** The mineralizing nodules were visualized by imaging the integrated area of the $\nu_3 \text{PO}_4$ band. **(b)** Second derivative (inverted) of the $\nu_3 \text{PO}_4$ band. Although the average of all spectra forming the image shows typical features of apatite (green line), the presence of ACP was clear in the spectra of some specific areas (red line). **(c)** Distribution of apatite based on the intensity of the second derivative peak related to mature mineral at $\sim 1015 \text{ cm}^{-1}$. **(d)** Distribution of ACP based on the intensity of the second derivative peak at $\sim 992 \text{ cm}^{-1}$. **(e)** Overlay of the distribution of apatite (green) and ACP (red), indicating the presence of apatite in the more developed core of the nodules and of ACP components mainly around their edges, in areas of nodules expansion and development. The color bars reflect lower (blue) to higher (red) intensity of the component of interest.

Bone mineral crystallization

Figure 8: Schematic of bone mineral crystallization and detection of the conversion of amorphous calcium phosphate (ACP) into apatite.

The conversion of ACP into apatite may involve different stages, in which atoms in the center of amorphous particles rearrange into a crystalline core where apatite is nucleated. As the mineral crystallizes, atoms from the ACP component are progressively incorporated into the crystal lattice, leading to the growth of the apatite domain and reduction of the amorphous shell. Here, we demonstrate that progressive stages of bone mineral crystallization may be identified by Fourier transform infrared (FTIR) spectroscopy, showing in the spectra the loss of the specific marker of ACP at $\sim 992 \text{ cm}^{-1}$ and increase of that of apatite at $\sim 1015 \text{ cm}^{-1}$.