Published in final edited form as:

Bone. 2015 February ; 71: 244–256. doi:10.1016/j.bone.2014.11.003.

Extracellular matrix mineralization in murine MC3T3-E1 osteoblast cultures: An ultrastructural, compositional and comparative analysis with mouse bone

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Abstract

Bone cell culture systems are essential tools for the study of the molecular mechanisms regulating extracellular matrix mineralization. Such *in vitro* studies provide insightful information on molecular determinants of mineralization, mineralization-related pathologies, tissue engineering and biomaterial technologies. In order for cell culture models to have relevance to the mineralization process as it occurs physiologically *in vivo*, it is imperative that the mineral crystals formed *in vitro* be of a correct phase, shape, size, orientation, location and crystallinity. MC3T3-E1 cell cultures are the most prevalent *in vitro* model of bone matrix mineralization. Despite their widespread use, there is as yet no systematic characterization of the mineral produced in these cultures, and mechanisms of extracellular matrix mineralization remain unclear.

In this study, we performed a comprehensive, multi-technique characterization of the morphology, composition and ultrastructure of cell culture biomineral to determine the suitability of MC3T3-E1 cultures for biomineralization studies, to establish definitive parameters of MC3T3-E1 culture mineralization, and to provide insight into important similarities and differences between culture mineral formed *in vivo* and *in vitro*.

X-ray diffraction (XRD) on intact (nonhomogenized), resin-embedded cultures indicated that similar to mouse bone, apatite crystals in culture grow with preferential orientations along the

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(100), (101) and (111) nucleating planes indicative of a guided biogenic growth as opposed to dystrophic calcification. XRD analysis of isolated culture crystals revealed that the mineral was a poorly crystalline hydroxyapatite with 10–20 nm-sized nanocrystallites. Consistent with XRD observations, electron diffraction patterns indicated that culture mineral was of a low crystallinity typical of biological apatites. As expected, energy dispersive x-ray spectroscopy of the culture mineral showed the presence of calcium and phosphorus, and trace amounts of sodium and magnesium from the mineral phase. Fourier transform infrared spectroscopy confirmed the presence of apatitic carbonate and phosphate. With all the techniques utilized, cell culture mineral and calvarial bone were remarkably similar and were distinguished from synthetic hydroxyapatite which, in contrast, displayed high crystallinity and large crystal sizes. Ultrastructural analysis with scanning and transmission electron microscopy showed that cultures have a dense, assembled collagenous matrix where mineralization proceeds with the appearance of small 100 nm collagen-associated mineralization foci which coalesce to form larger mineral aggregates. Furthermore, confocal imaging and three-dimensional reconstructions revealed that some cells exhibited dendritic processes and become embedded within the mineral in an osteocyte-like manner.

In summary, we have documented the characteristics of the mineral phase deposited in MC3T3-E1 cell cultures, and determined that the structural and compositional properties of this mineral are similar to that of mouse calvarial bone.

Keywords

Cell culture; Biomineralization; Hydroxyapatite; Osteoblasts; MC3T3-E1 cells

1. Introduction

In addition to studies on intracellular signaling and osteoblast differentiation, bone cell culture systems are essential tools for the investigation of the molecular mechanisms that regulate the mineralization of the extracellular matrix (ECM) [1]. Such studies provide valuable information for tissue engineering, biomaterial innovation, basic health research and for the therapeutic management of mineralization-related pathologies such as osteomalacias and ectopic calcifications. For *in vitro* studies to have any relevance, it is vital that the mineral formed and the mineralization process be of a physiological nature.

Although there are a multitude of material characterization techniques potentially applicable to the study of biomineral deposited *in vitro* (and also *in vivo*), there is little work in the literature involving systematic characterization of the biomineral formed in osteoblast cell cultures. Most investigators rely on classical histological methods using von Kossa or Alizarin red staining, even though these stains have repeatedly been shown to be insufficient for the accurate assessment of the mineral phase generated in cell cultures [2]. Limitations of such assays rest on the inherent nonspecific staining of these chemical reagents. For example, although the von Kossa reagent stains for phosphate, it does not specifically stain apatite mineral and may stain other mineral phases, just as it may potentially stain high concentrations of protein-bound phosphate in bone [3]. Likewise, Alizarin red chelates calcium and will stain calcium regardless of its source, potentially including extracellular matrix mineral, salts, precipitates, protein-bound calcium or even free calcium in solution

[4]. Moreover, these same chemical stains will stain nonphysiological dystrophic calcification [2].

In addition to the requirement for having an appropriate mineral phase, bone mineralization also requires correct crystallite size, orientation, location and quantity, a well-defined crystallinity that includes lattice substitutions, all occurring within a dense mature collagenous matrix. MC3T3-E1 cells are a murine calvaria-derived pre-osteoblastic cell line used as archetypal model of *in vitro* osteogenesis [5]. Following isolation and cloning of the MC3T3-E1 cell line on the basis of high alkaline phosphatase activity (an early marker of osteoblast activity), MC3T3-E1 cells were further subcloned based on their potential to mineralize and on their expression of late markers of osteoblast differentiation such as osteocalcin and bone sialoprotein [6]. The MC3T3-E1 subclone 14 is one such highly-mineralizing cell subclone, and is the most commonly used cell line in *in vitro* bone cell biology research.

Despite the wide use of the MC3T3-E1 cell line as a model of osteogenesis, the mechanism by which the extracellular matrix mineralizes remains unclear, and to date there is no systematic characterization at the ultrastructural level of the mineral that is produced in these bone cell cultures. To re-evaluate and definitively determine the suitability of MC3T3-E1 osteoblast cultures as a model for investigating molecular determinants of bone mineralization, we performed a multi-technique ultrastructural assessment of matrix-mineral relationships and extracellular matrix architecture in these cultures. The main objective of this manuscript thus was to perform a comprehensive characterization of the morphology, composition and structure of the biomineral produced by MC3T3-E1 osteoblasts *in vitro* to provide insight into potentially important similarities and differences between bone mineral formed *in vivo* versus that formed *in vitro* in order to establish parameters for MC3T3-E1 culture use in biomineralization studies. In addition, we aimed to improve our understanding of the relationship between the organic extracellular matrix and the biogenic mineral at the ultrastructural level. Taken together, this work provides information on the mechanism by which bone mineralizes, and describes the limitations and/or suitability of bone cell culture systems for investigating mineralization processes.

2. Materials and Methods

2.1 Cell culture set-up

MC3T3-E1 murine calvarial osteoblasts (subclone 14) cells [6] were received from Dr. R. T. Franceschi (University of Michigan, Ann Arbor, MI). The cultures were maintained in modified α -minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone)(selected from multiple lot/batch testing for being permissive of mineralization) and 1% penicillin-streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. All experiments were carried out at a plating density of 50,000 cells/cm². Cell differentiation and matrix mineralization were initiated 24 h after plating, with medium supplemented with 50 μ g/ml ascorbic acid (Sigma), and 10 mM β -glycerophosphate (Sigma) as a phosphate source enabling mineralization. Calcium was provided by 1.8 mM CaCl₂ being present in the basal media. The medium was changed every 48 h.

2.2 Minerals and preparations

2.2.1 Hydroxyapatite standards—Synthetic carbonated (7.7% w/w) hydroxyapatite powder (C-HA) was prepared following the aqueous precipitation method reported by Penel *et al* [7], except that ammonium salts were used (ammonium phosphate dibasic and ammonium carbonate). The carbonate level was determined by FTIR spectroscopy using a calibration set of carbonated apatites with carbonate content determined by elemental analysis. Hydroxyapatite standard reference powder material (HA-SRM) was from NIST (National Institute of Standards and Technology, Bethesda, MD, USA). Everywhere used in this manuscript the carbonated hydroxyapatite and hydroxyapatite standard reference materials are termed C-HA and HA-SRM, respectively.

2.2.2 Isolation of mineral crystals from MC3T3-E1 cell cultures—Cell culture mineral crystals were isolated following a modified protocol described previously [8]. Briefly, cell culture plates from day-12 mineralized osteoblast cultures were gently rinsed three times with deionized water (resistivity > 18 MΩ) followed by gentle spatula scraping from the culture dish surface, incubation in 1.3% sodium hypochlorite to remove organic components, and then a brief period of sonication. Crystals were pelleted at 5000 *g* and then immediately washed three times in double-distilled water before sequential washing with 50%, 95% and 100% ethanol. Crystals were stored in 100% ethanol at −20°C.

2.2.3 Isolation of mineral crystals from mouse calvaria—Mineral crystals from mouse calvariae were isolated using a procedure that does not alter the structure or composition of mineral crystals disaggregated from bone [8]. Briefly, all washing solutions were pre-equilibrated with synthetic hydroxyapatite for more than 24 hours. Calvariae from 3–4 week old Balb/C mice were first rinsed briefly with physiological saline and deionized water. The cleaned calvariae were then air-dried and hand-ground in an agate mortar. Ground bone (50–100 mg per 3 whole calvariae) was digested in 10 ml of 1.3% sodium hypochlorite at room temperature with continuous sonication for 10 min. Crystals were pelleted at 5000 *g* for 5 min, and then immediately washed three times in hydroxyapatite-equilibrated, deionized water and then sequentially double-distilled water before dehydration with 50%, 95% and 100% ethanol. Crystals were stored in 100% ethanol at −20°C.

2.3 Light microscopy and biochemistry of MC3T3-E1 cultures

2.3.1 Histology and quantification of mineralization—Mineral was visualized in culture wells by von Kossa staining using 5% silver nitrate solution (Sigma) followed by exposure to bright light, and by calcein staining where day 12 cultures were incubated in 25 ug/ml calcein in PBS for 1 hour and post-fixed in 4% paraformaldehyde. For quantification of mineralization by measuring amounts of deposited calcium, cultures were decalcified with 0.5 M HCl, and calcium levels in the supernatant were determined spectrophotometrically (wavelength, 595 nm) using a calcium assay kit (Diagnostic Chemicals) and a microplate reader.

2.3.2 Collagen deposition assay—Collagen matrix deposition was quantified by picrosirius red staining followed by stain dissolution/release with 0.1 M NaOH and

microplate reader spectrophotometric measurement (wavelength, 562 nm) as described previously [9]. Bovine calf skin collagen type I (Sigma) was used as a standard.

2.3.3 Immunolabeling for confocal optical microscopy—Cells were cultured on 8-well chamber slides, fixed in 3.7% formaldehyde, and permeabilized with 0.25% Triton X-100 in PBS. Labeling of the actin cytoskeleton was performed using phalloidin Alexa Fluor 564 (Invitrogen), and nuclei were marked using DAPI (4', 6-diamidino-2-phenylindole) (Invitrogen) staining. Collagen was immunolabeled using rabbit anti-collagen type I (Chemicon) and anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen). Fluorescence imaging was performed using an LSM 510 META Axioplan 2 confocal laser scanning microscope equipped with a C-Apochromat 40x/1.2 water-immersion objective (Carl Zeiss). Alexa Fluor 488, Alexa Fluor 564, and DAPI were excited with a 488-nm argon laser, a 543-nm helium-neon laser, and a tunable (740-nm, two-photon) pulsed titanium sapphire laser (Verdi V10/Mira900), respectively. Image stacks were processed using Huygens Professional 2.4 (Scientific Volume Imaging) and Imaris 4.0 (Bitplane) software.

2.3.4 Immunogold labeling for TEM—Ultrathin LR white acrylic resin sections (80 nm) were placed on Formvar-coated nickel grids. Post-embedding immunolabeling was performed on the grid-mounted sections as described previously using goat anti-osteopontin antibody (R & D Systems) and protein A-colloidal gold complex (15 nm; Dr. G. Posthuma, University of Utrecht, The Netherlands). Grids were conventionally stained for transmission electron microscopy using uranyl acetate and lead citrate. Images were recorded using a Philips FEI Tecnai 12 transmission electron microscope operating at 120 kV.

2.4 Ultrastructural characterization of MC3T3-E1 cultures

2.4.1 Electron microscopy, electron diffraction and energy-dispersive X-ray microanalysis—Cultures were fixed with 2% glutaraldehyde and dehydrated through a series of graded ethanol dilutions. For transmission electron microscopy (TEM), cultures were embedded in LR white acrylic resin or epoxy resin (Electron Microscopy Sciences). Ultrathin sections (80 nm thick) were placed on formvar-coated nickel grids and stained with uranyl acetate and lead citrate. A field-emission FEI Tecnai 12 TEM (Philips) was used to examine the sections at 120 kV, and electron diffraction was performed at 200 kV in the selected-area configuration. For scanning electron microscopy (SEM), cultures were dehydrated in hexamethyldisilazane, sputter-coated with Au-Pd or carbon, and imaged using an S-4700 field-emission gun scanning electron microscope (Hitachi High Technologies, Pleasanton, CA). Energy-dispersive X-ray was performed on SEM equipped with an INCA microanalytical system (Oxford Instruments, UK),

2.4.2 X-ray diffraction—X-ray diffraction analysis was performed using a Bruker D8 Discover diffractometer equipped with a copper X-ray tube (wavelength, 1.54056 nm), and a general-area detector diffraction system (GADDS). All components (X-ray source, sample stage, laser/video alignment/monitoring system and detector) are mounted on a vertical θ - θ goniometer. Measurements were run in coupled θ - θ scan in either conventional powder diffraction mode (500 μ m beam spot size) or the more localized area microbeam analysis

mode (50 μm beam spot size). The conventional powder-diffraction configuration was used to analyze standard control powder samples (hydroxyapatite standards C-HA and HA-SRM; analyzed as-received without any treatment), and mineral crystals isolated from cell culture and bone calvariae (pulverized by crushing/grinding). Microbeam mode was applied to characterize cell culture samples and bone calvariae embedded in epoxy resin blocks for small localized analyses of mineral-abundant regions identified on the block face. More details about the XRD are provided in the Supplemental Information section.

2.4.2 Fourier-transform infrared spectrometry—An attenuated total-reflectance Fourier-transform infrared spectrometer (ATR-FTIR, Spectrum 400, PerkinElmer Instruments) equipped with a ZnSe diamond-coated ATR crystal was used to analyze the various samples by infrared spectrometry. ATR-FTIR was performed in the transmittance mode at 4 cm^{-1} resolution over a range of 4000–650 cm^{-1} . Spectra were baseline-corrected for comparison (Spectrum software, Perkin-Elmer). The mineral phase was characterized by ATR-FTIR and samples were briefly washed in PBS, and frozen at -80°C and freeze-dried for further analyses.

3. Results

3.1 Matrix deposition and mineralization

Upon exposure to ascorbic acid which facilitates collagen fibrillogenesis, MC3T3-E1 osteoblastic cells produce and assemble an abundant extracellular fibrillar collagen matrix network (Fig. 1A, two left panels) that accumulates steadily within the first 6–8 days of culture (Fig. 1B). Without ascorbic acid treatment, osteoblasts still synthesize collagen but fail to externalize and assemble the collagen network (Fig. 1A, two right panels). During the last 6 days (of 12-day cultures), and in the presence of a phosphate source such as 10 mM β -glycerophosphate to enable mineralization, the matrix gradually mineralizes as determined by many methods, including histology methods with von Kossa (Fig. 1C) and calcein (Fig. 1D) staining (for mineral ions) and biochemical quantification of acid-extracted precipitated calcium within the cultures (Fig. 1E). von Kossa staining for phosphate revealed the formation of mineral nodules deposited within the cell/matrix layer as seen in the dishes or in histological cross- and *en face view*-sections (Fig. 1C), where some cells became embedded within the mineral (Fig. 1C, right panel) in an osteocyte-like manner. Confocal microscopy of calcein-stained mineralized cultures revealed an extensive interconnected network of mineral throughout the matrix of the cultures (Fig. 1D). Confocal microscopy of osteocyte-like cells embedded within matrix/mineral showed long and extended dendritic cell processes (Fig. 1F) similar to those seen *in vivo*.

3.2 Ultrastructural morphology and compositional characterization

Observation of the upper surface (uppermost side in direct contact with the culture media) of a 12-day mineralized culture by scanning electron microscopy (SEM) revealed a continuous layer of cells with numerous filopodia (dendritic cell processes), blebs and membrane projections (Fig. 2A). Individual cells within this confluent layer were flattened and roughly polygonal in shape. A cross-section of the culture dish observed by SEM revealed alternating layers of noncontiguous cell sheets embedded in a fibrillar organic matrix (Figs.

2A,B). The sheets of matrix within the culture were comprised of densely packed bundles of collagen fibrils (Fig. 2B) which by transmission electron microscopy (TEM) displayed the characteristic 64 nm banding periodicity typical of type I collagen fibrils (Fig. 2C). At sites of mineralization in the extracellular matrix, some collagen fibrils are encrusted with small globular masses having a diameter of approximately 100 nm (Fig. 2D). Backscattered-electron SEM imaging – where higher atomic number elements appear brighter – coupled with X-ray microanalysis indicated that these globular structures and adjacent mineralized collagen fibrils contained high levels of calcium and phosphorus (Fig. 2E). While often isolated, in many other areas the small globular masses appeared as coalesced masses representing large deposits of mineral. TEM of normal mouse bone (Fig. 2F) and mineralizing MC3T3-E1 cultures (Figs. 2G,H) showed similar ultrastructural mineralization features including larger mineralized areas that seemed to derive from growth of small mineralization foci/globules within the collagenous extracellular matrix. Mineralized collagen fibrils were also evident by TEM at most sites of mineralization, as well as very small (~150 nm size in diameter) nodules of mineral between collagen fibrils, all of which were strongly positive for osteopontin after immunogold labeling (Fig. 2I). These small nodules may correspond to the small globular masses observed by SEM in Figure 2D whereas the larger areas of mineralization may correspond to the aggregated globular masses observed by SEM in Figure 2E. The presence of osteopontin at these sites is indirectly indicative of mineralization events since this osteoblast-secreted noncollagenous phosphoprotein intimately associates with nascent and growing apatite crystals formed with the extracellular matrix [10, 11].

3.3 Mineral crystal characterization in MC3T3-E1 cultures

3.3.1 High-resolution TEM imaging and electron diffraction—High-resolution TEM (HRTEM) imaging of ultrathin sections of epoxy-embedded mineralizing MC3T3-E1 cell cultures revealed abundant apatite nanocrystals between and aligned with the collagen fibrils (Figs. 3A–C) that were similar to those seen in normal mouse bone (Fig. 3A, inset). Electron diffraction of the mineral shown in Figure 3C (inset) demonstrated a diffraction pattern characteristic of apatite, which was comparatively explored in greater detail in MC3T3-E1 culture mineral, and mouse bone mineral (in calvariae), as compared to powder control standards (Fig. 4). For this, the crystal structure of osteoblast culture mineral, calvarial bone, synthetic carbonated hydroxyapatite (C-HA) and hydroxyapatite standard reference material (HA-SRM) were examined by selected-area electron diffraction (SAED). SAED patterns of MC3T3-E1 cell cultures (Fig. 4A) and bone calvaria (Fig. 4B) were similar, with diffraction rings matching those of both carbonated hydroxyapatite (C-HA, Fig. 4C) and pure hydroxyapatite (HA-SRM, Fig. 4D) powders. Diffraction patterns from cell culture and bone mineral exhibited characteristic diffuse rings compared to the sharp distinct pattern of C-HA and HA-SRM, indicating that the culture and bone samples have lower crystallinity which is typical for biological apatites and that correlates with X-ray diffraction (XRD) observations (see below). The most intense diffraction rings in the culture and bone SAED patterns are attributable to unresolved, overlapped diffractions peaks from the mineral planes (211), (112), (300), and (202), which again was substantiated by the XRD observations (see below). For the synthetic apatite powders HA-SRM and C-HA, individual

diffraction spots align as rings which is indicative of mineral having high crystallinity and a significant content of relatively large single crystals.

3.3.2 X-ray diffraction of homogenized mineral samples—The crystal structure of the mineral formed in MC3T3-E1 cultures was assessed by X-ray diffraction (XRD) and compared to the mineral found in mouse calvaria, synthetic carbonated hydroxyapatite (C-HA) and hydroxyapatite standard reference material (HA-SRM). The biological crystals were isolated, homogenized, and organic components removed by NaOCl treatment as described in the Methods section. A control culture sample with organic matrix still present (NaOCl untreated) was also included for analysis. As shown in Figure 5, the cell culture mineral has a pattern characteristic of a poorly crystallized hydroxyapatite material, typical of that reported for biological apatites. The XRD peaks obtained from the culture mineral have 2θ positions matching those of a hydroxyapatite phase (Fig. 5 and Table 1). The broadness of the peaks indicates that crystallites are relatively small (10–20 nm in size as estimated by the Debay-Scherrer formula using peak width) and may exhibit a certain degree of imperfection (inter-planar distances with variations caused by intracrystalline impurities and substitutions). The most intense, well-resolved, distinct reflections from crystal planes (211), (112), (300) and (202) characteristic of a highly crystalline hydroxyapatite as seen in the HA-SRM powder appeared unresolved for the culture mineral, but they still appeared as single large peak in the 2θ 30.5°–35.0° range centered at 31.8°. This large peak indeed convolutes into the four reflections above.

A key observation from these XRD spectra is that the diffraction patterns from mineral of MC3T3-E1 cell cultures and mouse calvarial bone are remarkably similar (Fig. 5), with the main feature characteristic of these patterns being the broad peak overlapping the (211), (112), (300) and (202) reflections that exhibit essentially the same shape and centered peak 2θ position for both samples. The (300) and (202) reflections, being well-resolved for both HA-SRM and C-HA, were no longer demonstrable as distinct peaks for the cell culture mineral and the bone calvarial mineral; they had however distinguishable spectral shoulders at around 33° and 34° in 2θ , for HA-SRM and C-HA, respectively. Other intense reflections expressed as broad peaks found in both cell culture and calvarial mineral were identified as (100), (001), (310), (222) and (213). Collectively, these XRD data indicate that the mineral deposited *in vitro* by MC3T3-E1 cells have a similar crystal structure to the mineral phase of mouse calvarial bone.

Treatment of mineralized tissues with NaOCl removed most of the organic, noncrystalline organic material as is evidenced by comparison of the XRD spectra of the untreated culture to the NaOCl-treated culture mineral (Fig. 5). The untreated MC3T3-E1 spectrum exhibits a pronounced background 2θ bump originating from amorphous material such as the organic extracellular matrix. In addition, the pattern from the untreated sample exhibits high noise in the intensity signal along the whole 2θ scanned range characteristic for X-ray diffusion and scattering on unordered material. The XRD spectrum of the NaOCl-treated culture mineral has a significantly lower background and an improved (less noise) signal. Details of the 2θ positions and the interplanar spacing of the mineral crystals as determined from these XRD analyses are listed in Table 1.

3.3.3 X-ray diffraction of resin-embedded mineral samples—A major limitation of traditional XRD analysis of homogenized, ground mineralized tissues is the inevitable loss of information about the orientation of the crystals in their native state within the organic extracellular matrix. The process of crushing and grinding produces grain homogenization as grains displaced from their locations are rendered randomly oriented in the sample volume. This sample preparation/homogenization method, commonly and intentionally applied for powder material devoid of organics, has the advantage of allowing accurate identification of the phase of the material being analyzed. Biological samples on the other hand are essentially nonhomogeneous in both phase content and chemical composition.

To determine whether the mineral crystals in the MC3T3-E1 cell cultures and bone calvaria have preferred orientations within the sample, we performed XRD measurements on resin-embedded samples applying a microbeam capability to analyze locally mineral in very small areas that can be correlated with histological detail at the cell and matrix level. Figure 6 shows typical microbeam X-ray diffraction patterns from MC3T3-E1 cell culture mineral and from mouse calvarial bone, with both being embedded in resin blocks. The inset light micrographs in Figure 6 identify the X-ray microbeam spot size (50 μm in diameter, red circle) analysis site as determined from a single histology section cut from the block face prior to XRD microbeam analysis. Microbeam XRD diffraction peaks recorded for both cell culture mineral and bone calvarial mineral had Miller indices of (100), (101), (110), (200), (111), (210), (202), (222) and (213), with (101), (110) and (200) being the most intense (Fig. 6). Interestingly, not all the (hkl) reflections characteristic of a polycrystalline hydroxyapatite phase seen for the samples in form of powder (Fig. 5) were observed. The most intense peaks at (211) and (112) for polycrystalline hydroxyapatite seen in the powder pattern of Figure 5 did not appear in microbeam cell culture or calvarial block data (Fig. 6) – thus indicating that the apatite crystallites are not randomly oriented in the samples and that they have specific orientations in the analyzed sample volume. Moreover, the intensities of the diffraction peaks reflected by the planes (100), (101) and (111) were significantly higher than the intensities at which these peaks normally occur in hydroxyapatite powder. The presence of a preferential orientation of growing apatite crystals along the planes (100), (101) and (111) are underscored by the occurrence in the pattern of multiple peaks (doublets), (200), (202) and (222) from the same planes, respectively.

A second finding of the microbeam-XRD analysis is that apatite crystallites showed a nonhomogenous distribution across the depth of the sample. Thus, while at angle $\theta_1 = 10^\circ$ only peaks from (100) and (101) were detected in the $5\text{--}37^\circ$ 2θ range of the measured frame, at $\theta_1 = 25^\circ$ (deeper within the block) new peaks appeared as (110), (200), (222) (Fig. 5). This clearly indicates that the cell culture mineral deposition develops with hydroxyapatite crystals exhibiting specific orientations at specific depths within the sample. In comparison to calvarial bone, we observed that the culture mineral exhibited the same diffraction peak positions with some variation in peak intensity (Fig. 5). Together, these results indicate that apatite crystallites grow in cell culture with orientations and distributions similar to those grown in calvarial bone tissue.

3.4 Chemical composition

3.4.1 Energy dispersive X-ray spectroscopy—The chemical composition of the mineral samples was investigated by energy-dispersive X-ray spectroscopy (EDS) in SEM. Figure 7A shows representative EDS spectra of the MC3T3-E1 cell culture mineral, mouse calvarial bone, synthetic carbonated hydroxyapatite and hydroxyapatite standard reference material performed by on-face block microanalysis. As expected, the spectra show the presence of calcium and phosphorus, an elemental composition consistent with apatite, in all the samples (Fig. 7A). Trace amounts of sodium and magnesium were also detected in both cell culture mineral and the calvarial sample (data not shown); small amounts of such metal ions are often incorporated into biological apatites although contamination from culture media or sample processing buffers cannot be completely excluded. Other elements detected in both culture mineral and calvarial bone were carbon and oxygen which originate from the organic matrix and the embedding resin. The high signal intensity of the carbon and oxygen precluded a quantitative determination of the mineral Ca/P ratio.

3.4.2 Fourier-transform infrared spectrometry (FTIR)—The chemical composition and molecular environment of the matrix and mineral in the various samples were characterized by FTIR analysis. Figure 7B presents FTIR spectra of MC3T3-E1 culture mineral and control samples of calvarial bone, synthetic carbonated hydroxyapatite (C-HA) and hydroxyapatite standard reference material (HA-SRM). The biological samples (cell culture mineral and calvariae) were processed by homogenization and grinding as described in Materials and Methods. A culture sample, with organic components removed by NaOCl-treatment, was also included. All FTIR spectra show absorption bands characteristic of mineral, *i.e.* the phosphate group (PO_4^{3-}) in the 1150–900 cm^{-1} range. Triply degenerate asymmetric stretching modes ν_{3a} , ν_{3b} , and ν_{3c} , of the P–O bond of phosphate occur with peaks around 1100–1090 cm^{-1} , 1075–1055 cm^{-1} and 1020–1010 cm^{-1} , respectively [12]. The non-degenerate symmetric stretching ν_1 of the P–O bond of orthophosphate was assigned by the band peaking at $\sim 960 \text{ cm}^{-1}$. For the HA-SRM sample, PO_4^{3-} bands were fairly well-resolved and narrow, indicating a high crystallinity of the powder. The wider aspect of bands seen for cell culture and bone calvariae is related to the well-known lower crystallinity and smaller crystallites in these biological apatites, as was also observed by electron diffraction (Fig. 4) and XRD (Fig. 5).

In cell culture and bone calvariae, absorption bands characteristic of the organic matrix were observed as spectral peaks within the amide and carbonyl group range of 1750–1250 cm^{-1} (Fig. 7). Amide exhibits bands with peaks at $\sim 1650 \text{ cm}^{-1}$, $\sim 1550\text{--}1535 \text{ cm}^{-1}$, and $\sim 1240 \text{ cm}^{-1}$ that are here termed according to the literature as Amide 1, Amide 2 and Amide 3, respectively [13]. The carbonate content CO_3^{2-} of the apatite mineral is expressed by the ν_3 bending modes at $\sim 1450 \text{ cm}^{-1}$ and $\sim 1420\text{--}1400 \text{ cm}^{-1}$ and the ν_2 stretching mode at 875–870 cm^{-1} [14, 15] adjacent to the band characteristic of orthophosphate. The other vibrational modes characteristic to CO_3^{2-} , ν_3 ($\sim 1085 \text{ cm}^{-1}$) and ν_4 ($\sim 750\text{--}690 \text{ cm}^{-1}$), are not seen here because one (ν_3) is convoluted within the strong phosphate, while the other is very weak.

A very noticeable finding was that the FTIR spectra of cell culture (as-processed, untreated) and bone calvariae were very similar (position, shape and intensity of bands, and peaks). The amide bands occur only in cell culture (as-processed, untreated) and bone calvariae, but not in the synthetic apatites, and these were completely removed by NaOCl treatment to remove organics. Moreover, the presence of ν_3 carbonate modes of similar intensity and area in the treated cell cultures, and in calvariae and carbonated hydroxyapatite, indicates that this signal originates from structural carbonate in the apatitic lattice of the mineral. Removal of organic matrix by NaOCl treatment does not remove the carbonate peaks confirming that bone mineral also contains a carbonated hydroxyapatite phase.

The assignment and position of bands and peaks in the FTIR spectra for the analyzed samples are shown in Table 2.

Discussion

The *in vitro* culture of osteoblast cells is a convenient and useful method for the study of osteoblast activity that allows experimentation on biological processes related to bone formation in ways that are not always achievable in living organisms. Established cell lines provide a stable, homogenous and reproducible model to investigate factors affecting osteoblast signaling, differentiation and mineralization. Cell culture models that produce an abundant, collagen- and noncollagenous protein-rich extracellular matrix – such as occurs when appropriately using the MC3T3-E1 mouse pre-osteoblast cell line – are particularly useful in that they allow fundamental questions about matrix mineralization to be queried *in vitro*. While *in vitro* studies on biomineralization are routinely performed using other cell lines, frequently these investigations fail to verify the presence of a secreted and assembled extracellular matrix, and thus conclusions drawn from this work may not have physiological relevance to bone formation and mineralization as it occurs *in vivo*. The use of a wide variety of experimental methods to study biomineralization – whether performed *in vitro* or *in vivo* – provides the essential experimental armamentarium necessary to understand bone biology. All such methods are currently used to investigate normal bone formation and turnover, as well as various hyper- and hypomineralization (osteomalacia) pathologies, ectopic calcifications including in soft tissues, and tissue-engineered constructs and biomaterials. In this study, we focused on one widely used cell culture model that is thought to closely resemble in many ways bone formation and mineralization *in vivo*, particularly with regard to extracellular matrix secretion, assembly and mineralization. The work is timely in that to date, biomineralization studies have made wide use of this *in vitro* model system without there being a full and definitive mineral characterization of this cell culture model. Here, we have closed this information gap by using multiple analytical techniques to perform a comprehensive structural and compositional characterization of the biomineral and mineralization processes occurring in MC3T3-E1 osteoblast cultures, and have compared these data to that obtained from normal mouse bone tissue (the same species from which the culture model derived). In doing this comparison, we describe many key similarities between the two experimental models.

Of the analytical methods used, X-ray diffraction (XRD) remains the benchmark (“gold standard”) technique for the determination of crystal structure for any given mineral. While

a few previous studies have used XRD to demonstrate that MC3T3-E1 cultures are capable of depositing a bone-like hydroxyapatite inorganic mineral phase [16–18], those studies were limited by a sample preparation method that utilized lyophilized samples ground into powder prior to analysis. While this approach is very common for powder-cast materials, this grain homogenization treatment is not well-suited for mineralized biological samples which contain an abundant and relatively soft organic matrix, and which have mineral crystal orientation that provides key information on the biology and properties of mineralized extracellular matrices. Homogenization of samples by grinding into powders inherently involves detrimental destruction, disaggregation and mixing of sample components in a way that precludes access to information on important hierarchical tissue architecture and microscopic compositional organization and structure.

In this study, for comparative purposes, we repeated – like others have done previously – XRD analyses on dried ground/powdered homogenized samples. However, we additionally included various treatment steps, and we performed a careful and thorough analysis of intact resin-embedded samples of both mineralized cell cultures and bone tissue to allow us also to determine preferential crystal orientations and nucleating planes within the extracellular matrix. From the XRD performed on intact, resin-embedded samples, we demonstrate that the apatite crystals in the MC3T3-E1 cell culture possess preferential orientations within the volume of the sample analyzed – like they do for intact bone tissue – an observation arguing against more random dystrophic calcification. Unlike (hkl) reflections characteristic of polycrystalline powdered hydroxyapatite, XRD of resin-embedded MC3T3-E1 samples shows crystals with preferential orientation along the (100), (101) and (111) planes, with the (100) plane representing the prismatic faces of hexagonal hydroxyapatite prisms. These specific crystal orientations seen in both cell culture and in calvarial indicate guided biogenic crystal growth and not stochastic dystrophic calcification.

Remarkable similarity was found between the culture mineral and calvaria bone mineral using most of the other methods that we employed in our study. In both cases, the biogenic mineral was poorly crystallized and consisted of nanoscale-range crystals as evidenced by broadened peaks in X-ray diffraction and electron diffraction. Such observations are typical for biological hydroxyapatites where the poor crystallinity of the mineral is related to impurities, substitutions and accommodation of inorganic ions and/or small organic molecules within the crystals [19, 20]. Indeed, when compared to pure hydroxyapatite, bone mineral has carbonate substitutions within the lattice structure of the crystals, being substituted at approximately 7% at the PO_4 position, and to a lesser extent at the OH position [21]. Carbonate substitutions and other elemental impurities in bone (hydroxy)apatite increase crystal solubility thereby facilitating dissolution and resorption of bone during bone remodelling. In this study, elemental analysis of culture mineral by energy-dispersive spectroscopy (EDS, X-ray microanalysis) revealed an elemental signature consistent with that of calvarial bone, with FTIR spectroscopy confirming the presence of apatitic carbonate and phosphate groups. In *in vitro* studies, carbonated culture mineral has been observed by FTIR spectroscopy previously in rat bone marrow stromal cell cultures [22, 23], fetal rat calvarial cell cultures, chicken osteoblasts [24] and mouse 2T3 osteoblasts [2]. In *in vivo* studies, chick calvariae [25], cartilage [26], and bone tissue [27] have been studied by FTIR spectroscopy [28]. Few studies have performed FTIR spectroscopy on

MC3T3-E1 cultures or compared these spectra to bone – Luppen et al [16] examined matrix maturity and mineral crystallinity in MC3T3-E1 cultures under BMP2-stimulated or unstimulated conditions, but without a direct comparison to bone, and Bonewald et al. [2] failed to detect apatite mineral in MC3T3-E1 cultures by FTIR. In the latter case, the authors attributed this to perhaps changes in the cell line properties attributable to variations in tissue culture conditions or clonal selection. Indeed, we have shown that different serum lots commonly used for cell culture appear to contain variable levels of mineralization inhibitors that may inhibit extracellular matrix mineralization *in vitro*, [29] and thus it is common practice in our laboratory to screen for serum lots permissive for mineralization. Using such selected serum lot additives, along with ascorbic acid and β -glycerophosphate (regardless of dosing at 2, 4 or 10 mM; data not shown) as a phosphate source, culture conditions in our hands are highly permissive of what appear to be bone-like mineralization closely resembling physiological bone mineralization *in vivo* as described previously [6, 18][30–33] and in the current study. We thus encourage the use of the MC3T3-E1 cell culture model as an suitable approach to study bone extracellular matrix mineralization when appropriate culture conditions have been selected that are permissive to mineralization, and when collagen matrix secretion and assembly has been verified.

While this study was not designed to examine in detail the initial stages and locations of mineral deposition, our data show that the progression of mineralization in the extracellular matrix of MC3T3-E1 osteoblast cultures matches closely that observed in bone tissue. Concomitant with matrix mineralization in the cultures were the development of matrix-embedded, osteocyte-resembling cells showing numerous extended dendritic cell processes permeating the matrix and closely associating with extracellular mineralization foci (as shown in three-dimensions by confocal microscopy reconstructions). We also demonstrate ultrastructurally by SEM and TEM *in vitro* that these mineralization foci – like those found *in vivo* in native bone tissue – consist of agglomerations of apatitic crystals that align along collagen fibrils and that continue to propagate as larger mineralized areas throughout the matrix, all the while showing abundant accumulation of the mineralization-regulating protein osteopontin as occurs *in vivo* [34, 35].

Conclusions

In summary, we show that cultured MC3T3-E1 osteoblasts synthesize and assemble a collagenous extracellular matrix whose organization and mineralization occur in a manner very similar to that occurring in bone. We have determined that the mineral deposited in MC3T3-E1 osteoblast cultures occurs initially as small mineralization foci in the extracellular matrix, with subsequent propagation of mineralization throughout the matrix. Like for native normal bone tissue, the mineral phase in the cultures is a poorly crystallized, carbonate-substituted hydroxyapatite consisting of small nanocrystallites having specific orientations and preferential (100), (101) and (111) growing planes with their *c*-axis aligned parallel to collagen fibrils. From these findings and from the use of other biophysical approaches, we conclude that matrix-mineral relationships, and the mineral phase, are similar between MC3T3-E1 osteoblast cultures and mouse bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Lydia Malynowsky (McGill University, Montreal) for assistance with various microscopies, Line Mongeon (Facility for Electron Microscopy Research, McGill University, Montreal) for assistance with scanning electron microscopy, and Dr. David Liu for assistance with transmission electron microscopy. This work was primarily funded by the Canadian Institutes of Health Research (to MDM, and MDB and MTK), and in part by the Natural Sciences and Engineering Research Council (to SNN). MMT acknowledges support in part from NIH grant R01 AR47969. MDM, MTK, MDB and SNN are members of the McGill Centre for Bone and Periodontal Research, and the MDM and MTK are members of the FRQ-S Network for Oral and Bone Health Research.

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Highlights

- We characterize the mineral phase of the most commonly used *in vitro* model of bone mineralization.
- The mineral phase of murine MC3T3-E1 osteoblast cultures was compared to mouse bone.
- Biophysical methods used to characterize matrix and mineral and their relationships *in vitro* and *in vivo* included X-ray diffraction and microanalysis, Fourier-transform infrared spectroscopy, light and confocal microscopy, electron microscopy and electron diffraction.
- The mineral phase formed *in vitro* in MC3T3-E1 bone cell cultures is remarkably similar to that formed in mouse bone *in vivo*, thus justifying their use as a bone mineralization model.

Figure 1.

Collagen and mineral deposition in MC3T3-E1 osteoblast cultures. **(A)** Three-dimensional reconstructions of cells and extracellular matrix from confocal microscopy images (left two panels), and conventional immunofluorescence staining for collagen (right two panels), showing localization of collagen (green) and actin (red) at the initiation of culture (Day 0) and after matrix assembly (Day 6 and 10) with and without addition of ascorbic acid (AA) to facilitate extracellular collagen fibrillogenesis. In the AA-treated cultures, collagen is secreted by day 6 into the extracellular matrix and assembled into a fibrillar network. **(B)**

Picrosirius red staining and quantification of extracellular collagen in nontreated (NT) and AA-treated (AA) cultures. Collagen deposition was quantified by spectrophotometric absorbance measurements of picrosirius red stain release following dissolution, and is increased by the addition of AA. **(C)** Epoxy-resin histology section cut perpendicular (left panel) or parallel (right panel) to the culture dish surface of a 12-day mineralized culture showing mineral deposits stained black by von Kossa reagent (long arrows) and counterstained with toluidine blue. Inset shows the actual culture dish that was sectioned. Some cells (short arrows) become trapped within the mineralized matrix. **(D)** Three-dimensional reconstruction of calcein-stained mineralized matrix obtained by confocal microscopy at 12 days of culture showing an extensive interconnected mineralized network. **(E)** Calcium content in decalcified mineral extracts from β -glycerophosphate and ascorbic acid-treated cultures (β GP + AA) compared to untreated control cultures. **(F)** Three-dimensional reconstruction of the cell body and extended dendritic processes of a mineral-embedded MC3T3-E1 cell at 12 days of culture. Mineral is stained green with calcein and cell processes are stained red with phalloidin (for actin filaments). Nucleus is stained blue with DAPI. Dendritic cell processes permeate the mineralized matrix regions.

Figure 2.

Ultrastructural analysis of cell-matrix-mineral relationships in 12-day MC3T3-E1 osteoblast cultures by scanning (SEM) and transmission (TEM) electron microscopy. (A,B) SEM of culture cross-sections show the stratified organization of the culture where cells alternate with layers of abundant collagen fibrils. Cells at the uppermost surface of the cultures are flattened with some cell projections (inset) and form a generally continuous layer above alternating layers of osteoblast cell sheets (Ob) and extracellular matrix (asterisks). (C) TEM showing an osteoblast closely juxtaposed to collagen fibrils (asterisks) of the extracellular

matrix. CM, cell membrane; rER, rough endoplasmic reticulum; Nu, nucleus. **(D,E)** SEM of early mineralization foci (arrows) that appear as small globular masses amongst the collagen fibrils (asterisks) that in more mineralized areas merge and coalesce into larger aggregates of mineralized matrix. Backscattered electron imaging (BEI, panel E) of a region of heavy mineralization in the cultures attributable to the aggregation of many small globular masses illustrates the presence of high atomic number elements (whiter) within the small globular masses and within some collagen fibrils. X-ray microanalysis of the small globular masses confirm the presence of P and Ca (inset). **(F)** TEM of normal mouse calvarial bone showing mineralization foci (arrows) within the collagenous osteoid (asterisk). Ob, osteoblast. **(G,H)** TEM of decalcified MC3T3-E1 samples shows the spread of the mineralization beyond the early mineralization foci in the collagenous matrix (asterisk), and the accumulation of noncollagenous protein into these mineralized areas (arrows), here decalcified. Ob, osteoblast. **(I)** TEM and immunogold labeling for osteopontin in the collagenous extracellular matrix associating with the mineralized regions (arrow). Osteopontin is localized to the margins of calcified collagen fibrils, and to small mineralization foci (inset). Magnification bars equal 2.5 μm (A and inset, B), 0.2 μm (C) and 1 μm (D–I).

Fig 3A(Inset) - 0.5 μ m**Figure 3.**

TEM of apatite crystals in 12-day mineralized MC3T3-E1 cell cultures, and in mouse calvarial bone. (A) Overview TEM of apatitic nanocrystals (arrows) in unstained epoxy resin-embedded MC3T3-E1 cell culture, and in uranyl-stained mouse calvarial bone (inset) (asterisk, collagen fibrils). (B,C) Higher magnification TEM view of apatitic nanocrystals aligned longitudinally with collagen fibrils (brackets). The inset shows the electron diffraction pattern from this field of view, with diffraction maxima being characteristic for aligned apatite.

Figure 4.

Selected-area electron diffraction patterns with identified diffraction maxima of mineral in (A) 12-day MC3T3-E1 cell culture, (B) control mouse bone calvaria, (C) synthetic 7.7% carbonated hydroxyapatite (C-HA), and (D) synthetic pure hydroxyapatite NIST standard reference material (HA-SRM). The diffraction patterns of cell culture mineral and calvarial mineral are highly similar with diffraction maxima matching those of control hydroxyapatite powders. Diffraction rings are less-well defined (indicating comparatively less crystallinity) in the biological samples than in the control powder samples as is typical for biological hydroxyapatites.

Figure 5.

Conventional X-ray diffraction patterns as indicated for 12-day MC3T3-E1 cell culture mineral (including after treatment for removal of organic material), and for control samples of calvarial mouse bone, synthetic carbonated hydroxyapatite (C-HA) and synthetic hydroxyapatite standard reference material (HA-SRM). All samples were analyzed in powdered form, where C-HA and HA-SRM were used as received, and MC3T3-E1 cell cultures and bone calvarial samples were processed by drying, crushing and grinding of the original material. Miller indices of X-ray reflections from various (*hkl*) planes of the hydroxyapatite crystals are as indicated for HA-SRM.

Figure 6.

Microbeam X-ray diffraction patterns of 12-day MC3T3-E1 cell culture mineral and control calvarial bone as analyzed at a cut block surface after embedding in resin. Prior to X-ray diffraction analysis, a histological section was cut and stained with von Kossa reagent (staining black) for mineral (with counterstaining with toluidine blue, insets) to assist with X-ray microbeam placement (red circle with crosshairs, 50 μm diameter) on the block surface. Diffraction patterns were obtained with starting angle between the incident X-ray beam and the sample surface plane (θ_1) of 10 and 25 degrees.

Figure 7.

EDS, as recorded by on-face block microanalysis, and FTIR spectroscopy of 12-day MC3T3-E1 cultures (including NaOCl treatment to remove organic material), mouse calvarial bone, and powdered synthetic carbonated hydroxyapatite (C-HA) and synthetic pure hydroxyapatite NIST standard reference material (HA-SRM) (A) EDS spectra from all samples show abundant calcium and phosphorus. (B) FTIR analysis of powders from each sample show bands characteristic of hydroxyapatite mineral – phosphate as well as

absorption bands characteristic to of the organic matrix - amide and carbonate are evidenced for cell culture and bone calvaria.

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Table 1.

2 θ position and interplanar spacing of the (*hkl*) reflections for the 12-day MC3T3-E1 mineralized cell culture mineral (sample treated with NaOCl) and comparison with mineral in mouse calvarial bone. Control powders are synthetic 7.7% carbonated hydroxyapatite (C-HA) and synthetic pure hydroxyapatite NIST standard reference material (HA-SRM). Calculation of lattice spacing was done with an X-ray wavelength value of 1.5406 Å. The symbol '(sh)' denotes a diffraction peak that appears as shoulder (overlapped by a more intense adjacent peak).

Sample	MC3T3-E1 cell culture (treated)		Calvaria		Carbonated HA		HA SRM-NIST		HA - A.S.T.M. Card No. 9-0432
Miller Index	2 θ	d (Å)	2 θ	d (Å)	2 θ	d (Å)	2 θ	d (Å)	d (Å)
(100)	10.40	8.499	10.56	8.370	-	-	10.86	8.140	-
(101)	16.62	5.330	16.65	5.320	16.82	5.267	16.90	5.242	5.250
(110)	-	-	-	-	19.00	4.667	18.91	4.689	4.720
(200)	21.43	4.144	21.56	4.118	22.02	4.033	21.83	4.068	4.070
(111)	23.00	3.864	23.02	3.860	23.05	3.855	22.90	3.880	3.880
(201)	-	-	-	-	-	-	25.30	3.517	3.510
(002)	25.99	3.425	25.90	3.437	25.85	3.444	25.88	3.439	3.440
(102)	28.15	3.167	-	-	28.10	3.173	28.05	3.178	3.170
(210)	28.75	3.103	28.88	3.089	29.05	3.071	28.96	3.080	3.080
(211)	One large peak ~30-35 mean: 32.00 sub-peaks: 31.85 (sh) 32.10 32.25 33.00 (sh) 33.90 (sh)	2.795	One large peak ~30-35 mean: 32.00 sub-peaks: 32.10 31.80 (sh) 32.90 (sh) 33.90 (sh)	2.795	31.94	2.800	31.78	2.813	2.814
(112)					32.23 (sh)	2.775	32.17	2.780	2.778
(300)					33.00	2.712	32.90	2.720	2.720
					34.05	2.631	34.02	2.633	2.631
(301)	36.00	2.493	35.90	2.499	35.58	2.521	35.54	2.524	2.528
(212)	-	-	-	-	39.09	2.302	39.18	2.297	2.296
(310)	39.94	2.255	39.91	2.257	39.96	2.254	39.82	2.262	2.262
(221)	-	-	-	-	-	-	40.27	2.238	2.228
(311)	42.40	2.130	42.40	2.130	42.20	2.140	42.06	2.146	2.148
(302)	-	-	-	-	-	-	42.30	2.135	2.134
(113)	43.80	2.065	43.85	2.063	43.86	2.062	43.90	2.060	2.065
(400)	-	-	-	-	-	-	44.40	2.039	2.040
(203)	-	-	-	-	45.32	1.999	45.37	1.997	2.000
(222)	46.79	1.940	46.82	1.939	46.80	1.939	46.73	1.942	1.943
(312)	47.93	1.896	47.92	1.897	48.25	1.887	48.07	1.891	1.890
(320)	48.87	1.862	-	-	48.80	1.865	48.72	1.867	1.871
(213)	49.55	1.838	49.57	1.837	49.50	1.840	49.48	1.841	1.841
(321)	50.47	1.807	50.50	1.806	50.69	1.800	50.48	1.806	1.806
(410)	51.55	1.771	51.46	1.774	51.44	1.775	51.30	1.780	1.780
(402), (303)	-	-	-	-	52.21	1.751	52.10	1.754	1.754

Sample	MC3T3-E1 cell culture (treated)		Calvaria		Carbonated HA		HA SRM-NIST		HA - A.S.T.M. Card No. 9-0432
(004), (411)	53.28	1.718	53.21	1.720	53.25	1.719	53.22	1.720	1.722
(104)	-	-	-	-	-	-	55.50	1.654	1.684
(322), (223)	55.92	1.643	55.96	1.642	56.02	1.640	55.90	1.643	1.644
(313)	-	-	-	-	57.19	1.609	57.16	1.610	1.611
(501), (204)	-	-	-	-	58.35	1.580	58.15	1.585	1.587
(420)	-	-	-	-	60.10	1.538	59.90	1.543	1.542
(331)	-	-	-	-	60.45	1.530	60.42	1.531	1.530
(214), (421)	-	-	-	-	61.74	1.501	61.65	1.503	1.503
(502)	-	-	-	-	63.15	1.471	62.92	1.476	1.474
(510)	-	-	-	-	-	-	63.20	1.470	1.465
(304), (323)	63.92	1.455	63.90	1.456	63.96	1.454	64.03	1.474	-
(511)	65.70	1.420	65.68	1.420	65.37	1.426	65.05	1.433	-
(422)	-	-	-	-	-	-	66.47	1.405	-

Table 2.

Absorption bands and assignment of vibrational modes for FTIR spectra of the mineral samples (sh = shoulder).

Sample	MC3T3-E1 cell culture	MC3T3-E1 cell culture (treated)	Bone Calvaria	C-HA	HA-SRM	Assignment
Band / peak (cm ⁻¹)						
	1642	1640	1640	-	-	Amide I
	1536	-	1548	-	-	Amide II
	1452	1455	1452	1453	-	v3b, carbonate
	1406	1416	1405	1418	-	v3a, carbonate
	1240	-	1240	-	-	Amide III
	1115(sh)	1115(sh)	1115(sh)	1108(sh)	1090	v3a, phosphate
	1070(sh)	1074(sh)	1065(sh)	1060(sh)	1055(sh)	v3b, phosphate
	1013	1017	1014	1018	1020	v3c, phosphate
	960(sh)	960(sh)	958(sh)	966	964	v1, phosphate
	874	874	874	875	-	v2, carbonate