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Infrared spectroscopic characterization of mineralized tissues

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Abstract

Vibrational spectroscopy (Infrared and Raman), and in particular micro-spectroscopy and micro-spectroscopic imaging has been used to characterize developmental changes in bone and other mineralized tissues, to monitor these changes in cell cultures, and to detect disease and drug-induced modifications. Examples of the use of infrared micro-spectroscopy and micro-spectroscopic imaging are discussed in this review.

Keywords

Vibrational spectroscopy; Cell culture; Infrared and Raman

1. Introduction

The mineralized tissues found in vertebrates can be subdivided into those, which develop through normal physiologic processes (e.g., bones, teeth, calcified cartilage, etc.), and those that form through pathologic processes (e.g., atherosclerotic plaques, kidney and salivary stones, and other pathologic deposits). Table 1 lists examples of these two types of calcified tissues, and the mineral phase or phases they most frequently contain. As can be seen from the table, all the physiologic deposits contain an analogue of the naturally occurring mineral, hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$ (HA). With the exception of the enamel of teeth, all the other physiologically mineralized tissues are deposited upon a collagen matrix, while that of enamel is collagen-free. Because the spectra of these mineral components are quite distinct, vibrational spectroscopy (Raman and infrared) has been extensively used to study all of these tissues providing information on the nature of the mineral phases present, quantitative information on the changes in mineral and matrix composition as mineralization occurs, and the nature and amounts of substituents in the mineral (e.g., [1-7]). In accord with the theme of this issue, this review is focused on IR microscopy and microscopic imaging for characterization and diagnoses of normal and diseased mineralized tissues. Specifically, we shall examine developmental studies of bones and teeth using whole tissues and cell cultures, pathologic calcifications, and the study of bone disease.

In addition to the hyperspectral images (*x*–*y* position, *z* intensity or value of parameter in question) showing the distribution of the phosphate ν_1, ν_3, ν_2 carbonate or amide I peaks (Fig. 1), several calculated parameters have been validated for the HA-containing tissues. The ratio of the area of the ν_1, ν_3 phosphate vibration (900–1200 cm^{-1}) to that of the amide I vibration is directly related to the chemically determined mineral content (ash weight) [8,9]. Carbonate to amide I ratios or carbonate to phosphate ratios indicate the extent of carbonate incorporation in the hydroxyapatite lattice, and curve-fitting of the carbonate band reveals whether the

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carbonate has replaced hydroxide (A-type) or phosphate (B-type) in the apatite lattice [10]. The relative areas of sub-bands at 1060 cm^{-1} [11] or the ratio of the 1030 and 1020 cm^{-1} sub-bands [12] correlate linearly with the HA crystal size and perfection in the *c*-axis direction as determined by X-ray diffraction analyses. In IR imaging, this ratio is often expressed as a ratio of peak height intensities [13] because it is time consuming to curve fit the number of spectra in a single image, not to mention multiple images. The areas of sub-bands at 1660 and 1686 cm^{-1} (or their intensity ratios) is related to the amount of non-reducible as contrasted with reducible collagen-cross links [14,15]. Hyperspectral images enable visualization of each of these parameters in the systems under examination.

2. Characterization of the development of physiologically mineralized tissues

The formation of the mineralized tissues starts with the patterning of the skeletal elements [16] and proceeds through the differentiating and proliferation of the cells that synthesize the matrices upon which the mineral is deposited. Vibrational micro-spectroscopy has been used to describe the progression of mineralization in the developing tooth [17], the conversion of calcified cartilage into bone within the epiphysial growth plate [18-20], tendon calcification [21,22] and bone maturation [23-28]. Examining mice in which protein expression was ablated by gene-deletion (knockout) or enhanced by over-expression (transgenics) by vibrational micro-spectroscopy and imaging has allowed the impact of specific matrix proteins and cytokines to be evaluated. For example, deletion of transforming growth factor beta-1 was shown to result in a significant reduction in mineral content, mineral crystallinity and collagen cross-links in the secondary ossification center and cortical bones, consistent with a mechanism of impaired bone maturation in the TGF-beta-1 null mice [29]. Similarly, over-expression of the receptor for insulin growth factor 1 was shown to alter the pattern of mineralization [30].

The ablation or over-expression of genes for specific bone, cartilage and tooth specific matrix proteins in mice (Table 2, [31-39]) often produce mild changes in tissue phenotype which may not be readily detectable by standard radiological or histochemical techniques. Distinct effects on mineral content and mineral crystal size and perfection and collagen cross-link distributions can be determined by vibrational spectroscopic imaging. The changes noted in the majority of cases validate predictions made from solutionbased studies.

3. Characterizing the mineral in cell culture

Physiologic mineral deposition is regulated by cells. The cells produce the extracellular matrix (predominately collagen) that supports the deposition of the mineral and the non-collagenous matrix proteins that regulate the site and habit of the mineral crystals [40]. The cells also regulate the flux of ions and the synthesis of macromolecules that facilitate initial mineral deposition. While IR analyses of homogenized cultures can provide information on the characteristics of the mineral that is present in a homogenized culture (e.g., [41-47]), IR microscopy and imaging provides additional information by allowing visualization of the distribution of mineral and matrix at discrete sites within the culture [48-56]. This enables investigators to ask specific questions about temporal and spatial variations within mineralizing cultures and about the environments of specific cell types. The effects of genetic or chemical modulation of the cells and matrix in the culture systems can be monitored as the culture develops, and sites close to and distant from the modified cells.

To date while IR has been applied to bone forming cells (osteoblast cultures) [40-48], dentin forming odontoblasts [49-51] and calcifying cartilage cells (chondrocyte cultures) [52-56]. IR imaging has only been applied in a limited number of cases, in one to distinguish the different ways in which osteoblasts behave on different prepared surfaces that might eventually be used for tissue engineering [48], and in others to define the progression of calcification in differentiating mesenchymal cell cultures treated with the cytokine BMP-6 [56]. Fig. 2 shows

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some typical images of the mineral content, crystallinity and collagen cross-link ratio in mineralizing osteoblast and chondrocyte cultures. These images show that the mineral is deposited upon a collagen matrix, there is a gradient of mineral deposition and a gradient of crystal sizes. The advantage of such cultures is that temporal changes can be characterized without having to manipulate animals; the disadvantage is that these cultures do not completely mimic what happens in the body where there are a multitude of cells and metabolic processes that may have direct and indirect effects on mineralization.

4. Characterizing the mineral and matrix in pathologic calcifications

While IR or Raman examination of homogenized deposits is routinely used to identify the nature of the mineral deposit present [58-64], it often happens that there is more than one phase, and investigators are interested in which came first or in what the nature of the interaction is between the matrix and the deposit. We have recently applied IR imaging to the study of juvenile dermatomyositis, a disease in children in which calcific deposits form in muscle and fat. These deposits are generally surgically removed for relief of symptoms, and working with Dr. Lauren Pachman (Northwestern University), we not only demonstrated the presence of hydroxyapatite in these deposits, but we also noted the association of the mineral and lipid (Fig. 3), This feature would be missed in standard KBr pellets, where the matrix is often defatted before pellet preparation. The figure illustrates the type of information that can be learned from such diagnostic surveys. The vibrational spectroscopic image of a calcific deposit provides more information than routine histology, where separate stains must be used, and phase identification is often based on polarization studies of unstained sections. Where multiple phases are present, imaging may be the most rapid way of identifying which phases are in the center and periphery of deposits, rather than needing to dissect layers of the deposit for such analysis.

5. Characterizing the mineral and matrix in bone diseases

A recent concern in the bone community is the “quality of the bone” because it is believed to be most predictive of when a bone will fracture in patients with osteoporosis. According to a recent NIH consensus conference, “Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture. Bone strength reflects the integration of two main features: bone density and bone quality” [65]. The mechanical competence of bone and hence its risk of fracture are dependent on the bones' mass, architecture and material quality [66-69]. The IR parameters that may be related to bone quality are mineral content, mineral crystallinity and collagen cross-link ratio. IR images of normal bones show a broad distribution of these parameters while the distribution in osteoporotic bones is narrow, and mineral content tends to be decreased while the other parameters are increased (Fig. 4). IR imaging of bone biopsies thus becomes useful for diagnosing the quality of bone, above and beyond what can be learned from routine histologic studies [70-72].

Changes in bone quality in other diseases such as osteogenesis imperfecta [73-75], osteopetrosis [76], osteomalacia [77] and bone tumors [78] both prior to and after treatment may also be evaluated using these techniques. For example, the effects of alendronate treatment of a mouse model of osteogenesis imperfecta [79], parathyroid treatment of a monkey model of osteoporosis [80], and risedronate therapy in a rodent model of osteoporosis [81] have shown the alterations in mineral and matrix quality causes by these therapies. Similarly, there are a few studies of the effects therapeutics currently in use for the treatment and prevention of osteoporosis in human biopsies [82-85].

Each of these studies demonstrates changes in the spatial distribution of mineral and matrix properties in the biopsied tissues. These changes in the future may not only be used for

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diagnostic purposes, but also to select the most effective therapy for treatment of different bone diseases.

Uncited reference

[57].

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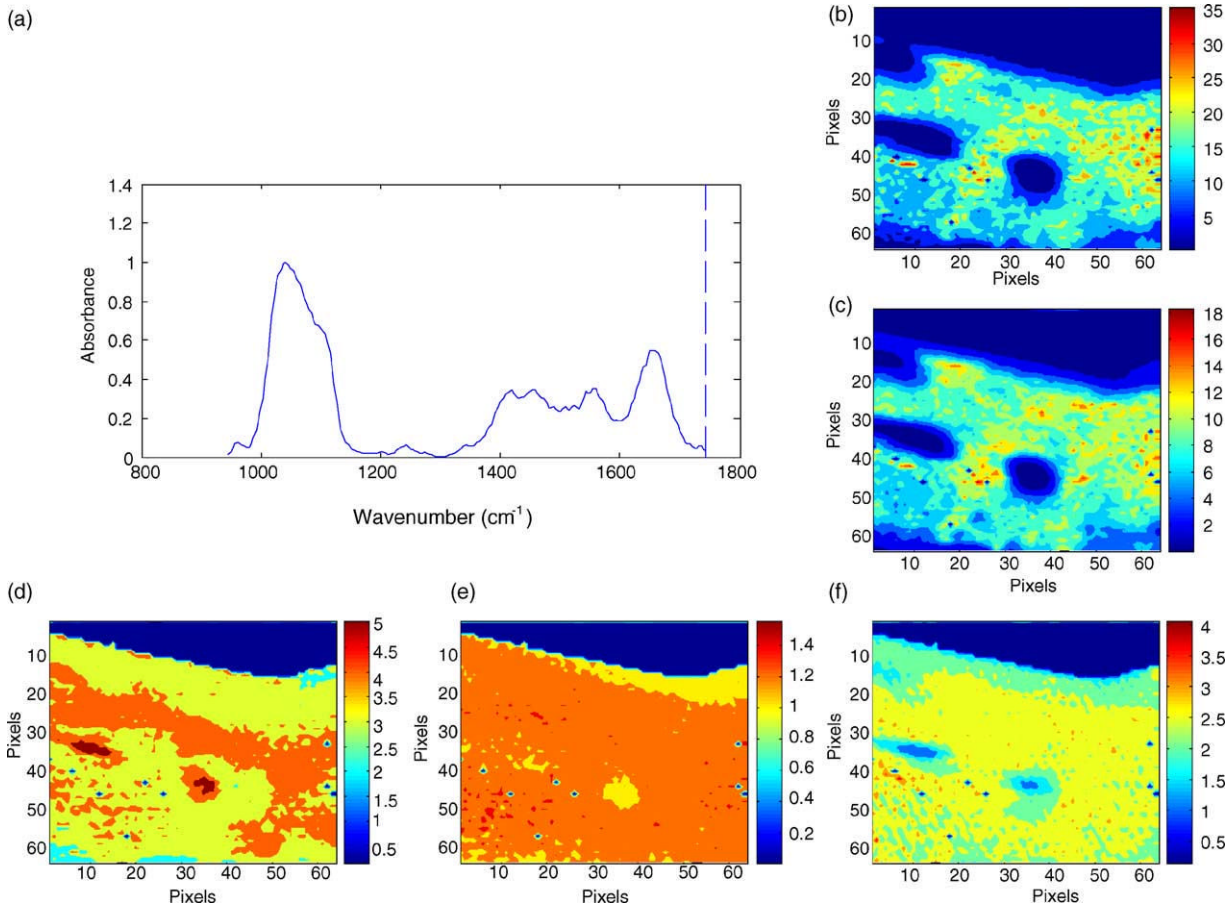

Fig. 1.

Hyperspectral images of bone mineral properties: in normal human cortical bone (a) typical spectrum from a single image pixel, (b) image of the mineral distribution in the biopsy, (c) image of the matrix distribution in the biopsy, (d) image of carbonate distribution, (e) image of mineral:matrix ratio, (f) image of crystallinity and (g) image of collagen cross link ratio. *Note*: all images are corrected for the presence of the embedding media, PMMA.

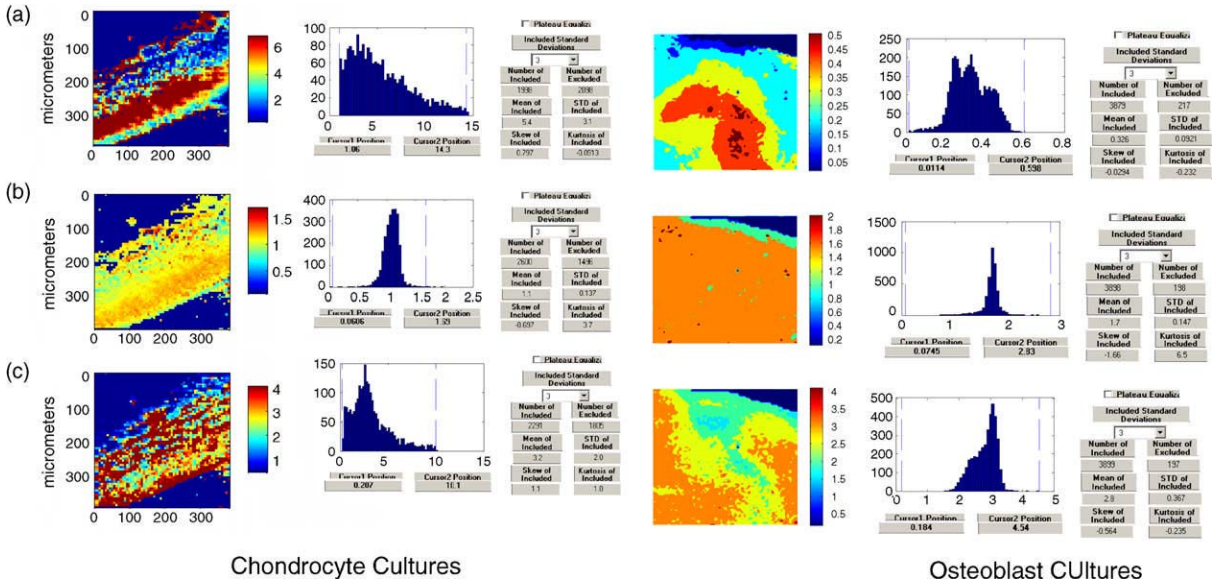

Fig. 2.

Images of mineralizing chondrocyte (left) and osteoblast (right) cultures: (a) mineral:matrix ratio, (b) crystallinity and (c) collagen cross-link ratio.

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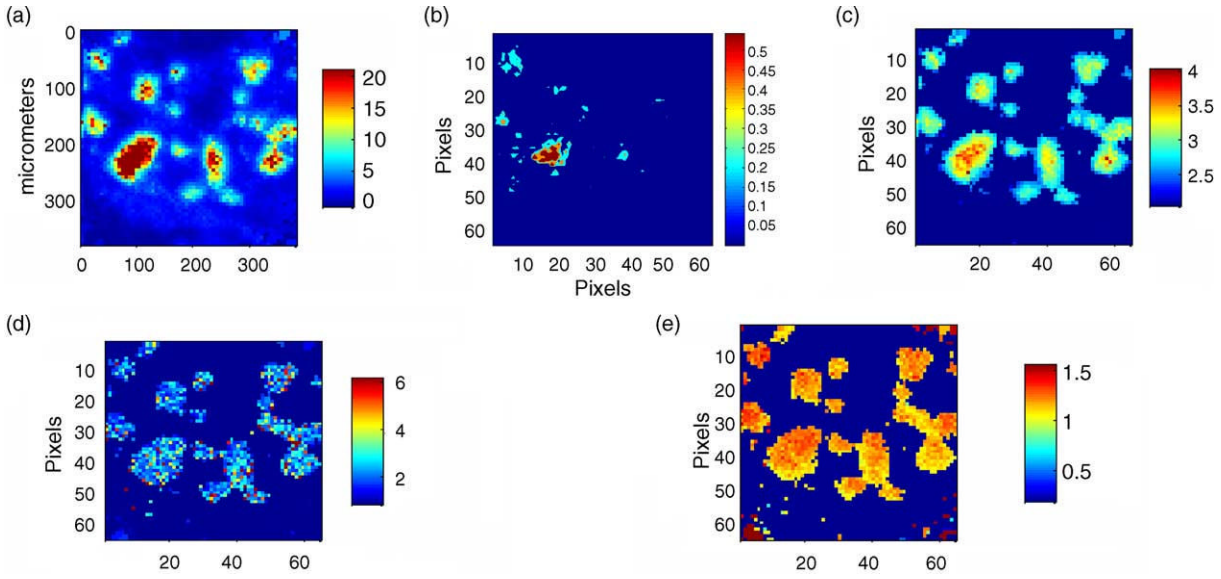

Fig. 3.

Image of a pathologic deposit removed from a patient with juvenile dermatomyositis: (a) mineral distribution, (b) lipid distribution, (c) mineral:matrix ratio (d) collagen cross-link ratio and (e) crystallinity distribution.

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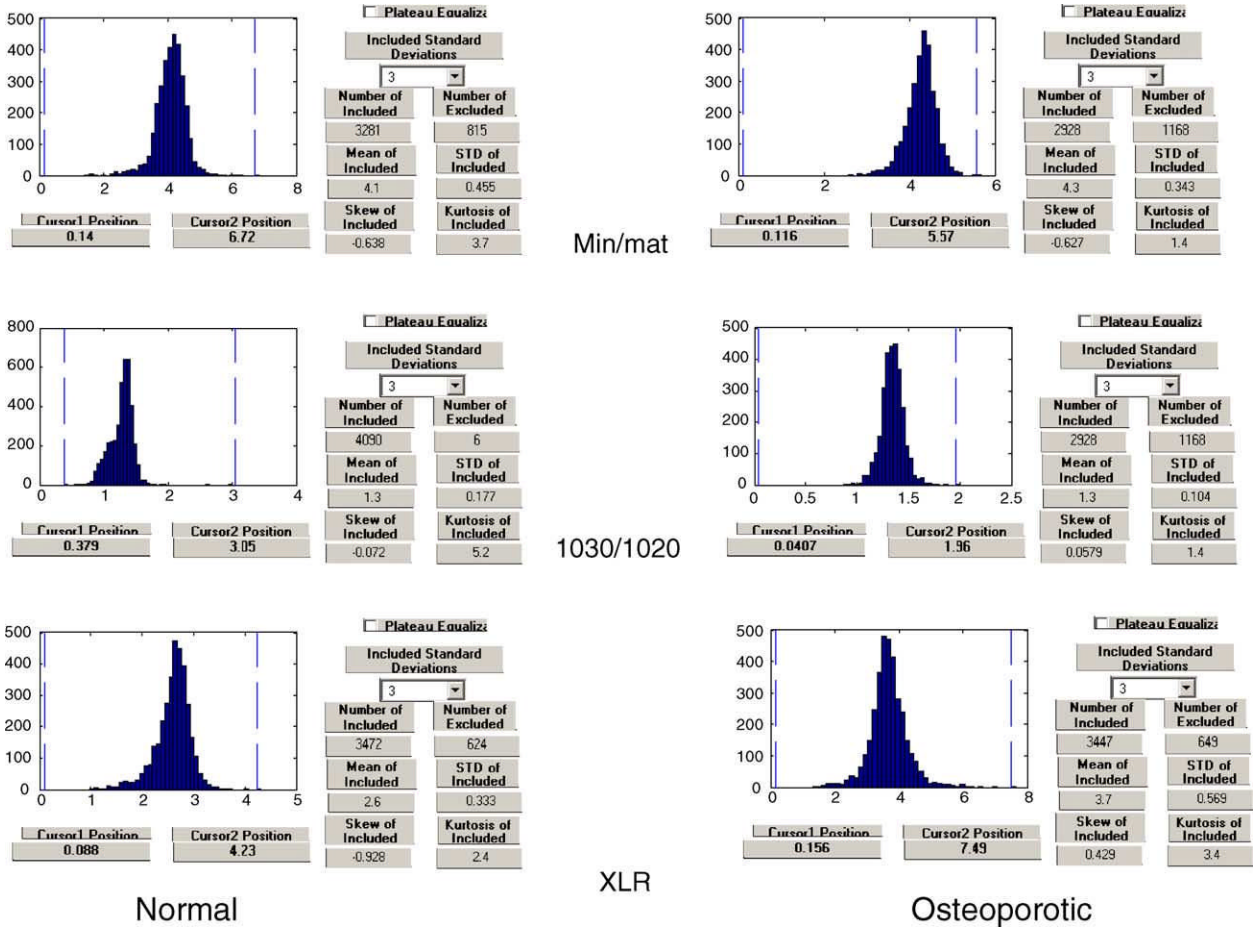

Fig. 4.

Pixel historgrams for the average distribution of mineral:matrix, crystallinity and collagen cross link ratio in normal and osteoporotic biopsies.

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Table 1

Vertebrate calcified tissues

Physiologic			Dystrophic		
Tissue	**Mineral**	**Matrix**	**Tissue/disease**	**Mineral**	**Notes**
Bone	Hydroxyapatite	Collagen	Atherosclerotic plaques	Hydroxyapatite	Lipid involvement
Calcified cartilage	Hydroxyapatite	Collagen	Prosthetic heart valves	Hydroxyapatite	Collagen based
Tendon and ligament insertions	Hydroxyapatite	Collagen	Tumoral calcinosis	Hydroxyapatite	Juxta-articular space
Cementum	Hydroxyapatite	Collagen	Juvenile dermatomyositis	Hydroxyapatite	Muscle and fat deposits
Dentin	Hydroxyapatite	Collagen	Milk alkali disease	Hydroxyapatite	Vitamin D toxicity
Enamel	Hydroxyapatite	Amelogenin enamelin	Kidney stones and salivary stones	Calcium oxalate, whitlokite, hydroxyapatite	
			Thalessemia	Iron oxides	Skin deposits related to transfusions
			Articular cartilage and intervertebral disk deposits	Calcium pyrophosphate dihydrate, monosodium urate, hydroxyapatite, calcium oxalate	

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Table 2

Knockout and transgenic mice evaluated by vibrational spectroscopy reveal significant variations from wildtype animals

Protein	Genetic modification	Technique used	Observed changes relative to age and background matched wildtype	Ref.
Type X collagen	Minigene insertion	FTIR-MS	Disordered mineral distribution	[30]
Type X collagen	Knockout	FTIR-MS	Disordered mineral distribution, no change in crystallinity	[32]
Osteocalcin	Knockout	FTIR-MS	Increased mineral content with no change in crystallinity (older animals only)	[34]
Matrix gla protein	Knockout	FTIR-MS, FTIRI	Increased mineral content, increased crystallinity	[84]
Biglycan	Knockout	FTIR-MS	Decreased mineral content, increased crystal size	[33]
Type I collagen	Natural mutation	FTIR-MS	Decreased mineral content, increased acid phosphate content	[73, 79]
Type I collagen	Knockin/transgenic	Raman	Age dependent changes in mineral content but not crystallinity account for mechanical adaptation	[38]
Osteopontin	Knockout	FTIR-MS	Increased mineral content, increased crystallinity	[35]
Osteonectin	Knockout	FTIR-MS, FTIRI	Higher mineral content, greater crystallinity, increased collagen cross links	[36]
Dentin matrix protein 1	Knockout	FTIRI	Decreased bone mineral content, increased crystallinity and collagen cross-link ratio	U
TGF-beta	Knockout	FTIRI	Decreased crystallinity, collagen cross-link ratio, nd mineral content in cortical bone and secondary ossification center	[29]
IGF 1-binding protein	Transgenic	FTIRI	Decreased bone mineral content and collagen cross-link ratio without change in crystallinity	[85]
Dentin sialo-phosphoprotein	Knockout	FTIRI	Decreased bone mineral content, increased crystallinity and collagen cross-link ratio	U
Vitamin D receptor	Transgenic	FTIR-MS	Decreased mineral content, no change in other parameters	[37]
Tissue specific alkaline phosphatase	Knockout	FTIR-MS	Decreased mineral content	[39]

FTIR-MS: FTIR micro-spectroscopy; FTIRI: FTIR imaging; U: unpublished.