Journal of Biomedical Optics 5(3), 259–268 (July 2000)

Application of vibrational spectroscopy to the study of mineralized tissues (review)

Angela Carden
Michael D. Morris
Department of Chemistry
University of Michigan
Ann Arbor, Michigan 48109-1055

Abstract. The infrared and Raman spectroscopy of bone and teeth tissues are reviewed. Characteristic spectra are obtained for both the mineral and protein components of these tissues. Vibrational spectroscopy is used to study the mineralization process, to define the chemical structure changes accompanying bone diseases, and to characterize interactions between prosthetic implants and tissues. Microspectroscopy allows acquisition of spatially resolved spectra, with micron scale resolution. Recently developed imaging modalities allow tissue imaging with chemical composition contrast. *© 2000 Society of Photo-Optical Instrumentation Engineers.* [S1083-3668(00)01203-X]

Keywords: Raman; infrared; bone; tooth; collagen; hydroxyapatite.

Paper JBO-90054 received Oct. 12, 1999; revised manuscript received Apr. 21, 2000; accepted for publication Apr. 21, 2000.

1 Introduction

Despite the constant expansion of medical knowledge, the processes governing many of the body's systems remain mysterious to researchers. The human skeleton is no exception. Bone is a complex system with many variables affecting its composition, formation, strength, and failure properties. The macroscopic processes that are easily observed are supported by microscopic processes that have not been seen directly. Many methods used to study bone have been inadequate to explore fully all the interactions that take place in such a dynamic system. In addition, most of these methods are unable to give information at the molecular level.

In the last decade or so, vibrational spectroscopy, both infrared (IR) and Raman, has been employed by an increasing number of researchers in the study of bone. Vibrational spectroscopy has the considerable advantage of being sensitive to both the mineral and organic components of bone, thus allowing for the study of mineral-matrix interactions as well as each individual component's properties. Sample preparation is relatively simple for both types of spectroscopy—once the specimen is excised and sectioned, no further preparation is necessary (though the sections must be thin for IR absorbance measurements). This nondestructive approach allows for spatial distribution mapping of bone's components as well as compositional analysis. Furthermore, traditionally prepared light microscopy specimens may also be examined using either Raman or IR spectroscopy, allowing for correlative studies. These spectroscopic techniques thus help to provide a complete picture of bone's composition unattainable by other commonly used methods such as x-ray diffraction or nuclear magnetic resonance (NMR). Most importantly, IR and Raman spectroscopies enable studies at micron-scale spatial resolution. The diffraction-limited spatial resolution achievable when using IR spectroscopy is 10–20 μm at the wavelengths of interest in bone studies, while the shorter wavelengths examined using the visible excitation of Raman spectroscopy yield a diffraction-limited spatial resolution of 1 μm or less. These fine spatial resolutions allow the observation of phenomena occurring at the microscopic level; these phenomena can then be used to help explain the macroscopic observations made using more traditional techniques. Finally, both IR and Raman spectroscopy are useful in the study of abnormal or diseased bone, providing clues to the causes and effects of such diseases as osteoporosis and osteogenesis imperfecta.

This review gives an overview of recent IR and Raman studies of mineralized tissues. Because IR spectroscopy is a better-established technique, a far more extensive literature exists for IR studies than for Raman studies. IR spectroscopy has been used to establish quantitative metrics for bone composition and crystallinity as well as being used for more qualitative studies. Raman spectroscopy, on the other hand, has found more applications in qualitative studies requiring a better spatial resolution, and in studies involving specimens that are unsuitable for IR studies because they cannot be made into thin sections.

The topics to be covered include some general background on bone and the two types of spectroscopy, as well as an examination of some considerations to be made when preparing specimens. This is followed by an overview of the application of vibrational spectroscopy to various fields, including the study of biocompatible implants; comparisons with synthetic apatites; mineralization; teeth; diseased and aging bone; and archeological applications. The developments in the relatively new area of vibrational spectroscopic imaging of bone and mineralized tissues will also be discussed.

2 Physiology and Chemistry of Bone

Bone tissue is a composite of an organic matrix and an inorganic mineralized component. The organic matrix is 85%–90% type I collagen fibrils, which provide a supporting matrix upon which the mineral crystals grow. Minor proteins provide additional structural strength as well as regulatory and signaling functions. The mineral fraction of bone is a carbonated

Address all correspondence to Michael D. Morris. Tel: 734-764-7360; Fax: 734-764-8815; E-mail: mdmorris@umich.edu

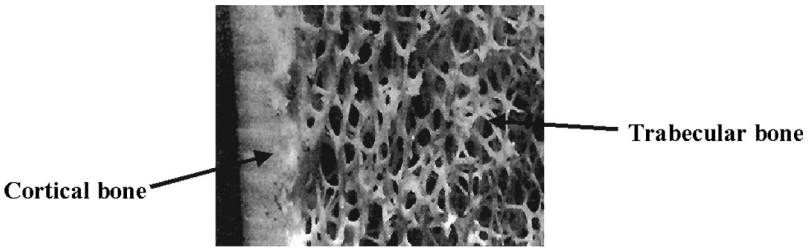

Fig. 1 Cortical and trabecular bone.

calcium phosphate, commonly described as apatitic. Varying amounts of other ions such as fluoride, chloride, and magnesium can also be incorporated into the crystal lattice, depending on the tissue's chemical environment and history.[1]

In the human skeleton, two types of bone exist. Cortical bone is a hard, dense tissue found on the outside of long bones. Trabecular bone is a spongy, less dense bone found inside the ends of long bones and inside vertebrae (Figure 1). While both types of bone contain the same basic components, the processes by which the two types of bone develop and are regenerated are quite different. Cortical bone is formed as osteoblasts tunnel through the bone in a cone-like structure which then fills in with circular lamellae, forming channels known as osteons. Trabecular bone forms and remodels in a notably different fashion; structures at its surface known as trabecular packets control the formation process which results in a porous open structure rather than the lamellar osteonal structure observed in cortical bone.[1] Because of the extensive variation between the various bone types, experimental methods that yield highly comprehensive information with minimal difficulty are desirable. Vibrational spectroscopy is an extremely useful tool in this endeavor.

3 Infrared Spectroscopy

The advantages of using infrared spectroscopy for studies involving biological specimens are evident. It is a well-established, well-understood technique, and there are many commercial systems available in several different configurations. Because of IR spectroscopy's larger spot size and fast acquisition times, it is very useful for studies in which spatial averaging is acceptable or desirable. IR spectroscopy also has the advantage that using it on biological specimens entails no problems with fluorescence. However, there is significant interference from water; its spectrum must be subtracted out or otherwise corrected for when working with hydrated specimens.

4 Raman Spectroscopy

Raman spectroscopy is also of great use in many biological applications. Its use of visible-wavelength excitation sources gives it better spatial resolution than that achievable by IR spectroscopy, allowing the study of variations on a smaller spatial scale. Since it is a scattering rather than an absorbance technique, Raman spectroscopy is not limited to transparent samples and can be used in several different configurations, though the most commonly used is a microprobe. Furthermore, Raman spectra exhibit little interference from water, making Raman spectroscopy advantageous for the study of many biological specimens.

Examples of both IR and Raman spectra of bone are shown in Figure 2. Table 1 summarizes IR and Raman band assignments and positions for bone tissue.

5 Special Considerations for Vibrational Spectroscopy

5.1 Specimen Preparation

Both Raman and IR spectroscopy are flexible where specimen preparation is concerned. Generally, specimens that are prepared for standard light microscopy will also work for vibrational spectroscopic studies. Some care must be taken not to perform procedures that may alter the bone's chemical structure and produce artifacts. One technique that is of particular concern is specimen fixing. In many studies, bone specimens are fixed to prevent bacterial growth and degradation of the specimen before data are collected. This naturally leads to concerns that specimens thus treated (and potentially chemically altered) are not truly representative of bone as it exists in the body. Fourier transform IR (FTIR) spectroscopy has been used to examine the effect of tissue fixation, both in formalin and ethanol, on the mineral and protein components of bone.[2] The researchers found that formalin fixation had little effect on the matrix proteins, but a measurable effect on the mineral structure, while ethanol fixation had the opposite effect, indicating that the choice of fixative should be carefully considered, depending upon what bone fraction is under scrutiny.

Specimen mounting is another area of concern, particularly for IR studies, where thin, transparent specimens are often required. In order to section bone thinly, the sample must be mounted in some sort of solid support, again raising concerns about the validity of studies on such samples. Pleshko and co-workers used FTIR to confirm that the effect of polymethylmethacrylate mounting medium on the IR spectrum of bone was negligible, indicating no chemical interaction between the bone and the medium.[3] However, when examining the physical or mechanical properties of bone rather than its chemical composition, clearly a mounting medium would not be advisable. Researchers also should be aware that many mounting media have their own vibrational spectral signatures, and these must be accounted for when using mounted specimens.

5.2 Specimen Fluorescence

Traditionally, Raman spectroscopy has had one major disadvantage when studying biological specimens: the green lasers

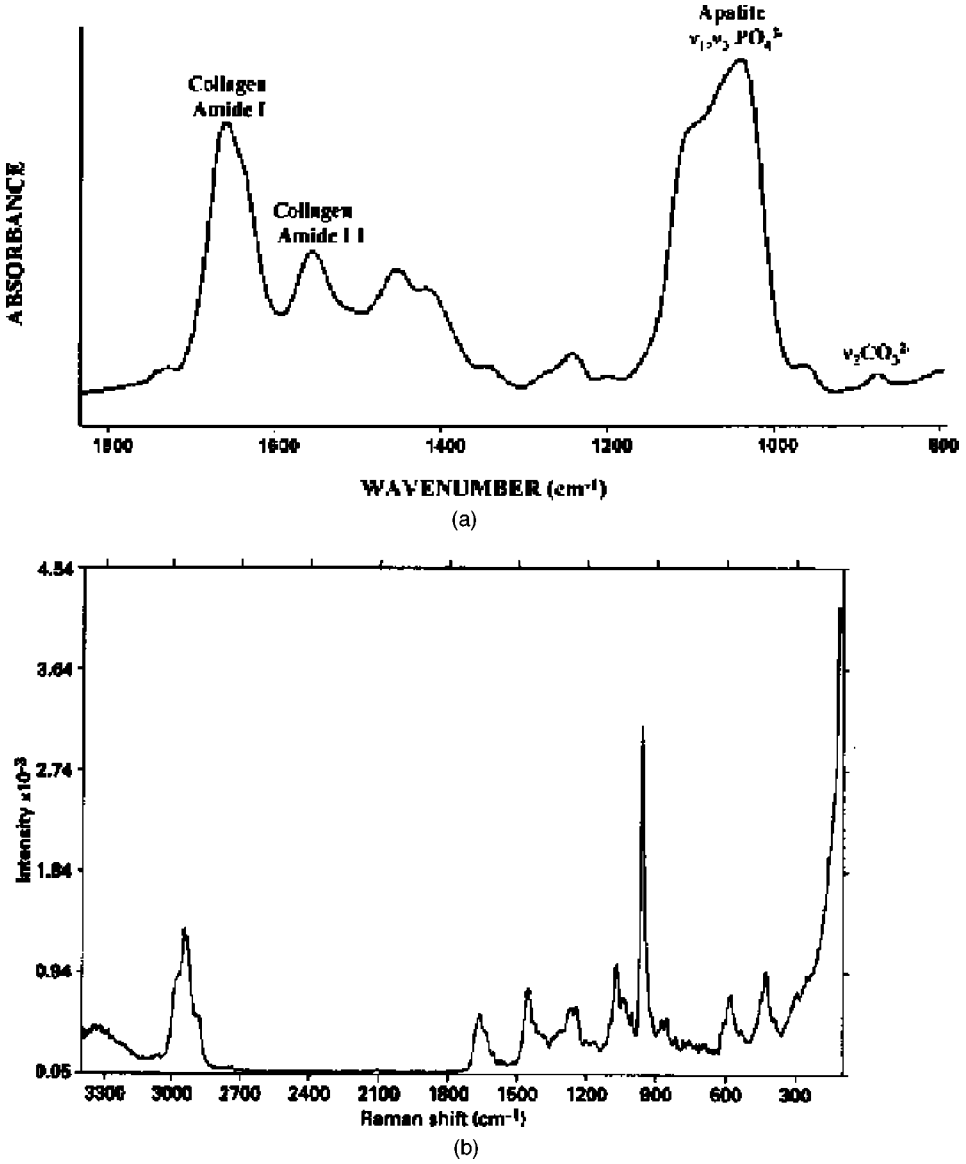

Fig. 2 Typical infrared (a) and Raman (b) spectra of bone tissue. Reprinted from Camacho et al., ''Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy,'' *Bone* **25**(3), 287–293, copyright 1999, with permission from Elsevier Science (a) and from Smith and Rehman, ''Fourier transform Raman spectroscopic studies of human bone,'' *J. Mater. Sci.: Mater. Med.* **5**, 775–778, copyright 1995, with permission from Kluwer (b).

that are most commonly used in Raman studies cause bone proteins to fluoresce, giving a background several orders of magnitude higher than the Raman signal. As a result, several protocols have been developed to deproteinate bone specimens in order to better observe the bone peaks. Frequently, the bone is simply deproteinated using hydrazine,[4] which effectively removes the organic matrix, leaving the mineral behind. Recently Penel and co-workers published a bleaching technique using hydrogen peroxide that appears to also be effective in reducing fluorescence without dissolving the organic matrix.[5] The Penel procedure has not been validated, however, and there is some concern in the biomedical community that any deproteination procedure may affect the structural and/or chemical properties of the bone by altering the properties of the fraction of the bone that remains after processing.[6]

Recently, the advent of near-IR Raman spectroscopy has made the study of biological specimens more feasible. Using deep red excitation eliminates much of the fluorescence and allows even the weak organic matrix bands to be seen clearly, while still maintaining the spatial resolution advantage inherent to Raman spectroscopy.[7–9] No deproteination is necessary.

6 Biocompatibility of Prosthetics and Implants

An area of active research in the Raman field is the study of bone's interaction with the coatings (usually calcium phosphate-based) on bone implants. Raman spectroscopic comparisons of the mineral in bone with synthetic hydroxyapatite have been done by Rehman et al.[4,7] and Penel et al.[10] In fact, Raman spectroscopy has been used to predict the biocompatibility of various implant coatings.[11] The results of this

Table 1 Raman and infrared spectroscopic band assignments for bone.

Assignment	Wave number range (cm^{-1})	Observed in	
PO_4^{3-} ν_2	422–454	Raman	
PO_4^{3-} ν_4	578–617	Raman/IR	
C–C stretching	815–921	Raman	
CO_3^{2-} ν_2	860–890	IR	
PO_4^{3-} ν_1	957–962	Raman	IR envelope
HPO_4^{2-} ν_3	1003–1005	Raman	
PO_4^{3-} ν_3	1006–1055	Raman	
CO_3^{2-} ν_1	1065–1071	Raman	
Amide III	1243–1269	Raman	
CH_2 wag	1447–1452	Raman/IR	
Amide II	1540–1580	IR	
Amide I	1595–1720	Raman/IR	
CH_2 stretching	2840–2986	Raman	
OH stretch	3572–3575	Raman	

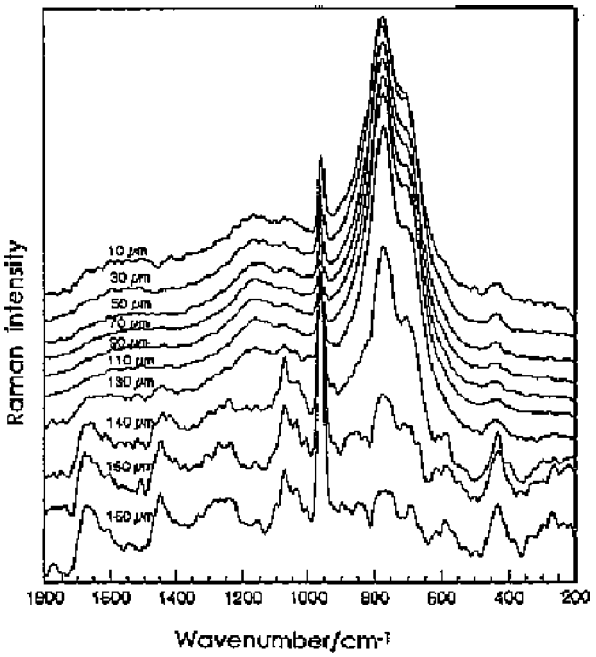

Fig. 3 Series of Raman spectra showing the progression from implant coating (top) to ongrown bone (bottom). Reprinted from Schrader et al., ''NIR Raman spectroscopy in medicine and biology: Results and aspects,'' *J. Mol. Struct.* **480–481**, 21–32, copyright 1999, with permission from Elsevier Science.

study showed that pure hydroxyapatite, a common implant cement and coating, was in fact not the best choice in terms of biocompatibility, due to the lack of bone growth precursors such as octacalcium phosphate (OCP) and dicalcium phosphate dihydrate (DCPD). Dippel and co-workers used FT-Raman spectroscopy to investigate the integration of bone into the implant coating[12] (Figure 3). De Grauw et al. have examined the crystallinity of these coatings, focusing on the width of the $\sim 960\ cm^{-1}$ phosphate peak as an indicator of the degree of crystal organization.[13] A more recent study by Penel and co-workers focused on brushite cement as a bone grafting material.[14]

In all these studies, bone mineral, which is frequently described in the literature as hydroxyapatite, has been found to possess qualities different from hydroxyapatite itself. Bone mineral shows evidence of HPO_4^{2-} in addition to apatitic phosphate, and is more amorphous than synthetic hydroxyapatite. Indeed, the appearance of a bone spectrum is comparable to the appearance of a spectrum of a carbonated apatite, which displays an increased full width at half maximum for the phosphate peak compared to pure hydroxyapatite.[15]

7 Ion Environments

Because bone mineral is not truly hydroxyapatite, there are variations in the ionic lattice. These substitutions are of interest to researchers since variations in the crystal structure may influence bone strength and other structural properties. Rey et al. used FTIR spectroscopy to look more closely at the different types and environments of phosphate ions in bone and enamel. Using band deconvolution of the ν_3[16] and ν_4[17] regions, they characterized the varying environments of the phosphate ion in bone and tooth enamel. The studies of the ν_4 band revealed the existence of a labile phosphate species as well as evidence of HPO_4^{2-} species. Examination of the ν_3 band suggested the presence of at least two nonapatitic phosphate species in immature bone, indicating the possible existence of bone precursors. Extensive studies have also been carried out on the different environments of the carbonate ion in a variety of specimens, including synthetic apatites,[18] tooth enamel,[19] and bone.[20] These spectroscopic data are supported by the information previously discovered by crystallographers regarding carbonate substitution into apatitic lattices. The main methods of substitution include exchanging with an OH^- ion (''A-type carbonation'') or exchanging with a PO_4^{3-} ion (''B-type carbonation''). Labile carbonate has also been found to be present. The various types of carbonation have been found to have distinctly different IR bands (Figure 4). In an extension of these studies, Pleshko et al. used synthetic hydroxyapatite as well as biological apatites to determine parameters for apatite crystallinity from infrared spectra.[21] Camacho and co-workers have used these and other parameters to compare data obtained from FTIR microspectroscopy to that obtained from scanning small angle x-ray scattering and have found excellent correlation between the two methods, once again validating the use of vibrational spectroscopy as a bone characterization technique.[22]

8 Mineralization

There is great interest in the chemistry of the mineralization process in bone. Mendelsohn and colleagues used FTIR microscopy to examine the mineralization gradient in growth plates, comparing normal rat femurs with rat femurs with de-

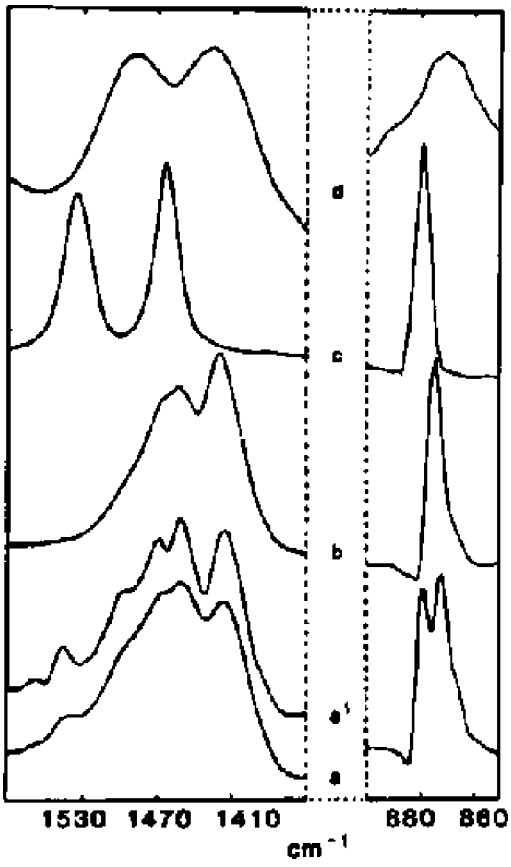

Fig. 4 Deconvolved infrared spectra of synthetic apatites showing different carbonation sites. (a) Type AB carbonate; a^1: type AB carbonate (ν_3 CO_3^{2-}); (b) type B carbonate; (c) type A carbonate; (d) amorphous carbonate. Reprinted from Rey et al., ''The carbonate ion environment in bone mineral: A resolution-enhanced Fourier transform infrared spectroscopy study,'' *Calcif. Tissue Int.* **45**, 157–164, copyright 1989, with permission from Springer.

fective endochondral ossification. The differences in mineralization, as shown by the intensity of the bands in the phosphate envelope, from the articular cartilage zone through the hypertrophic zone were clearly observable in the IR spectra, as were the differences in mineralization between the normal and abnormal specimens.[23] FTIR spectroscopy has also been used by Boskey et al. to study mineralization in chick limb bud mesenchymal cell cultures[24] as well as bone and cartilage[25] (Figure 5). In these studies a change in the size of mineral crystallites with increasing mineralization was observed, allowing the development of quantitative measurements of crystal size using infrared spectroscopy.

The role of proteins in the mineralization process has been studied as well, usually using genetically engineered mice. FTIR spectroscopy has been used in these types of studies to examine the role of osteocalcin[26] as well as leukemia inhibitory factor (LIF) and oncostatin M (OSM).[27] Osteocalcin-deficient mice were found to have less mature mineral (as determined by crystallite size) than normal mice, while LIF and OSM were found to contribute significantly to the short-range organization of the mineral phase, suggesting that these two cytokines play an important role in mineral phase development.

9 Teeth

Raman spectroscopy has been used frequently to study teeth. Dental enamel is a more crystalline, more highly oriented mineral than the mineral found in bone, but has a similar composition. Tsuda and Arends performed polarized Raman studies on dental enamel crystallites to examine the extent of crystal orientation.[28] They were able to see a distinct polarization dependence in their Raman spectra, comparable to that observed in a crystal of pure hydroxyapatite (Figure 6). In a separate study, the researchers used Raman spectroscopy to compare enamel with dentin, a different tissue also found in teeth, containing more organic matter than enamel.[29] These studies also compared various deproteination approaches, finding that each approach they tried caused the appearance of various spectral artifacts, including an induced increase in carbonation and decreases in band intensities.

A few infrared teeth studies also exist. An infrared study by Bottero, Yvon, and Vadot used a multivariate data analysis approach to examine the chemical variance between impacted and erupted teeth both with and without caries.[30] Using normalized principal components analysis, the investigators found that there was a correlation between the type of tooth (i.e., decayed or not, impacted or not) and such chemical characteristics as the tooth's composition and packing structure. FTIR photoacoustic phase analysis has been investigated by Sowa and Mantsch as a depth profiling technique for intact teeth.[31,32] The researchers were able to see carbonate and crystallinity gradients in dental enamel without sectioning the tooth—a possible solution to one of the main drawbacks of IR studies, namely the need for thin sections.

10 Diseased and Aging Bone

Infrared spectroscopy has been particularly useful for the comparison of bone that has been altered by disease or aging with normal bone. These comparisons allow researchers to correlate the physical properties of the altered bone with its chemical makeup, providing valuable insight into the pathology of diseases as well as information about the factors contributing to bone strength and healing.

10.1 Diseased Bone

In general, the most common comparison experiments involve the study of bone diseases. Because most bone diseases involve some modification of either the mineral or the organic portion of the bone, vibrational spectroscopy can give valuable information into the alterations occurring at a chemical level. Osteogenesis imperfecta (OI), or ''brittle bone disease,'' is one such disease. People suffering from this genetic disease do not possess the genes to express appropriate amounts of collagen. As a result, their bones, lacking a fully supportive organic matrix, are brittle and prone to fracture. IR spectroscopy has been used to compare bones from transgenic, induced-OI mice with bones from normal mice.[33] The results from this experiment showed no obvious differences between the composition of transgenic mouse bone and normal mouse bone, leading to the conclusion that the physical structure rather than the chemical makeup of the bone contributes to the effects of this disease. A similar disease, Schmid metaphyseal chondrodysplasia, has also been studied in transgenic mice. This disease involves mutations of the

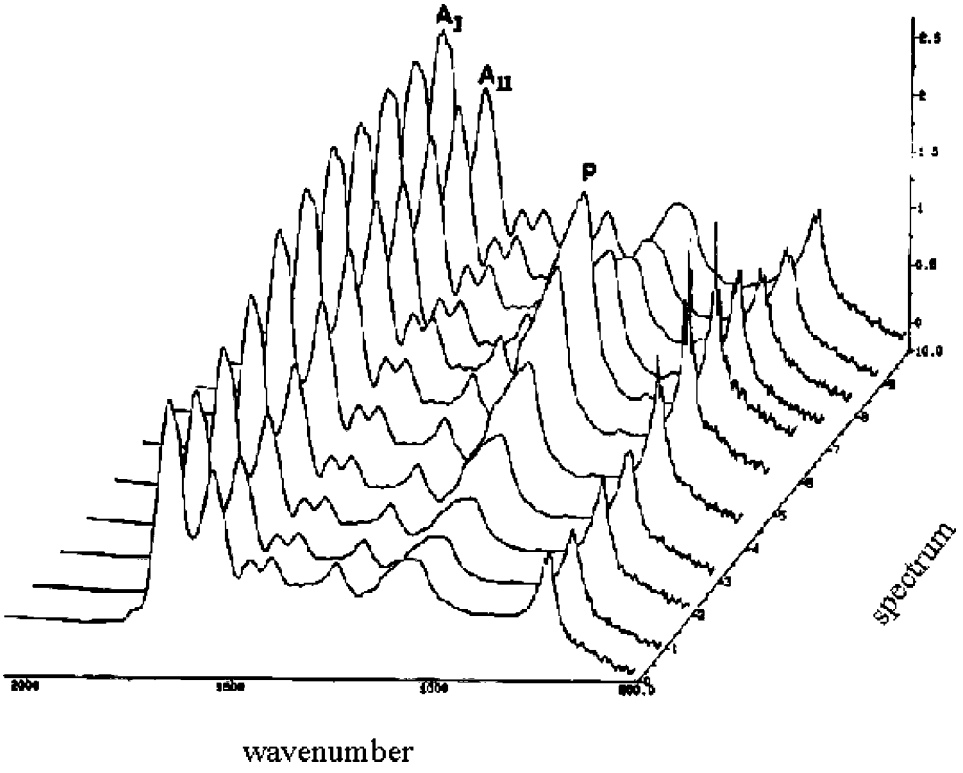

Fig. 5 Infrared spectral map showing the mineralization gradient in a cartilage nodule from a chick limb bud mesenchymal cell culture. Spectrum 1: nodule center. Spectrum 10: outer edges of nodule. Reprinted from Boskey et al., ''FT-IR microscopic mappings of early mineralization in chick limb bud mesenchymal cell cultures,'' *Calcif. Tissue Int.* **51**, 443–448, copyright 1992, with permission from Springer.

genes that produce type X collagen; the mutated collagen thus formed causes stunted growth and bowleggedness in children. Paschalis and co-workers have shown, using FTIR microscopy coupled with second derivative and curve-fitting analyses, that the mineral in mice transgenic for type X collagen is different in quality from that in normal bone, being even further from the normal apatite composition than bone mineral normally is.[34] In yet another mouse model of a human disease, X-linked hypophosphatemia, it was demonstrated using IR spectroscopy that the changes in the mechanical properties of diseased bone were correlated to not only the presence of less mineral, but a change in quality of the mineral that remained.[35]

An obvious disease to study is, of course, osteoporosis. Unlike the diseases discussed above, which involve changes to the bone's organic matrix, osteoporosis involves the weakening and/or loss of bone mineral. Paschalis et al. have demonstrated that osteoporotic cortical bone contains mineral that is more crystalline than that found in normal cortical bone.[36] In fact, osteoporosis seems to involve an advanced aging of bone that would normally be considered immature; for example, bone found near the center of an osteon.

10.2 Aging

In related experiments, IR spectroscopy has been used to study alterations in bone microstructure due to aging. An early study coupling IR data with x-ray diffraction data indicated that total carbonate content, bone crystallinity, and the

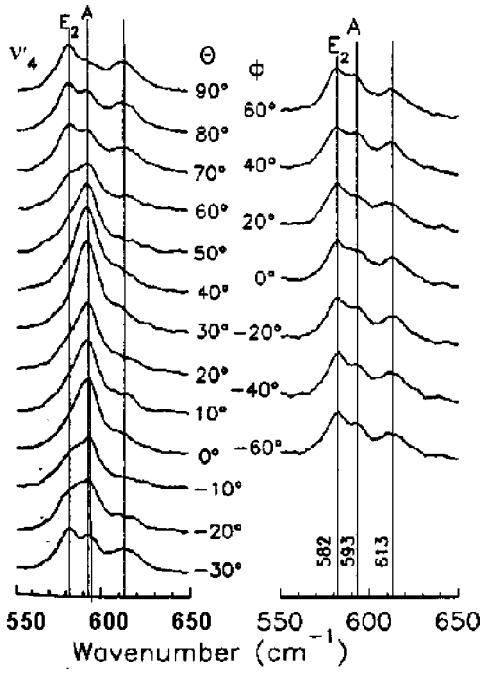

Fig. 6 Raman spectra of human tooth enamel showing polarization dependence of the phosphate ν_4 bands. Reprinted from Tsuda and Arends, ''Orientational micro-Raman spectroscopy on hydroxyapatite single crystals and human enamel crystallites,'' *J. Dent. Res.* **73**(11), 1703–1710, copyright 1994, with permission from Professional Publication Producers, Inc.

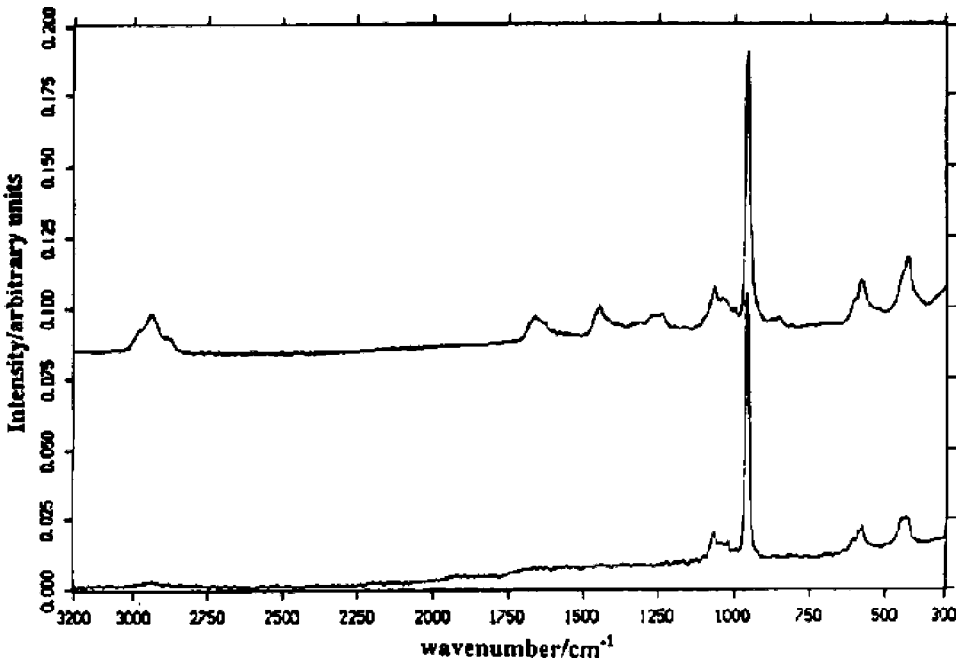

Fig. 7 FT-Raman spectra of modern tooth (top) and 4th century tooth (bottom) showing the loss of organic matter with interment. Reprinted from Kirchner et al., ''Ancient and modern specimens of human teeth: A Fourier transform Raman spectroscopic study,'' *J. Raman Spectrosc.* **28**, 171–178, copyright 1997 John Wiley & Sons Limited. Reproduced with permission.

ratio of calcium to phosphorus all increased with the age of the bone.[37] More recently, an examination of chicken bones of different ages has revealed that it is the unstable carbonate fraction that increases with age, while the ratio of A-type to B-type carbonate remains constant.[38] It has also been found that bone aging causes small positional and intensity shifts in bands associated with the amide I and II vibrations, possibly due to conformational changes in the proteins that make up the organic matrix.[39]

11 Archaeological Specimens

Interestingly, vibrational spectroscopy has even found applications in the field of archaeology. Because of its nondestructive nature, Raman spectroscopy has a distinct advantage over many characterization methods when working with one-of-a-kind specimens. Raman spectroscopy has mostly been used for the purposes of comparison between fossilized and modern teeth.[40,41] These studies have found that the mineral composition of interred teeth changes in subtle ways, becoming less carbonated with longer interment. In addition, the organic material in teeth appears to degrade or disappear with time; older specimens contain far less organic matter than modern specimens (Figure 7). Bertoluzza et al. have used this phenomenon to establish preliminary spectroscopic parameters for the dating of fossilized specimens, observing that the ratio of the CH_2 stretch at $\sim 2940\,cm^{-1}$ to the PO_4 stretch at $\sim 960\,cm^{-1}$ appears to correlate well with the age of the specimen.[42]

IR spectroscopy has had more limited application in archeological studies, perhaps because in the most commonly used experimental mode, transmission, it is necessary to slice the sample thinly. However, IR spectroscopy has been used to examine the degree of preservation of both fossilized teeth and bone dentin by Michel and co-workers.[43] Diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy has also been used to examine paleontological specimens,[44] with similar results.

12 Imaging

While point spectroscopic techniques can yield valuable information, the results gained from these studies lack a spatial context. Because bone is not a homogeneous substance, one point spectrum yields very little information about the bone as a whole. A spatial context is needed for these results to be validated. Recently, the use of vibrational imaging has expanded into the bone research arena, enabling the addition of spatial information to the preexisting spectroscopic data. Early Raman imaging efforts included the use of point mapping for the study of teeth[45] as well as line-scan imaging, using either a scanning mirror or an $x-y$ translation stage, for the study of bone implant coatings[46] and the interface of tooth dentin with adhesive.[47] Recently, the collection of three-dimensional (two spatial and one spectral) hyperspectral Raman images has become possible. Timlin et al. have used hyperspectral Raman imaging combined with multivariate analysis techniques to examine the phosphate gradients in cortical and trabecular bone[48] (Figure 8), and have also employed Raman transects, or lines of point spectra, to examine the tissue immediately around an osteon.[49,50]

Like Raman imaging, infrared imaging has only recently been applied to the study of mineralized tissues. Paschalis and co-workers used pointmapping techniques to build up spatially relevant band intensity images of human bone, using both a serpentine approach for human cortical and trabecular bone[51] and a radial approach to examine individual osteons.[52] Recently, infrared imaging has been accomplished using a

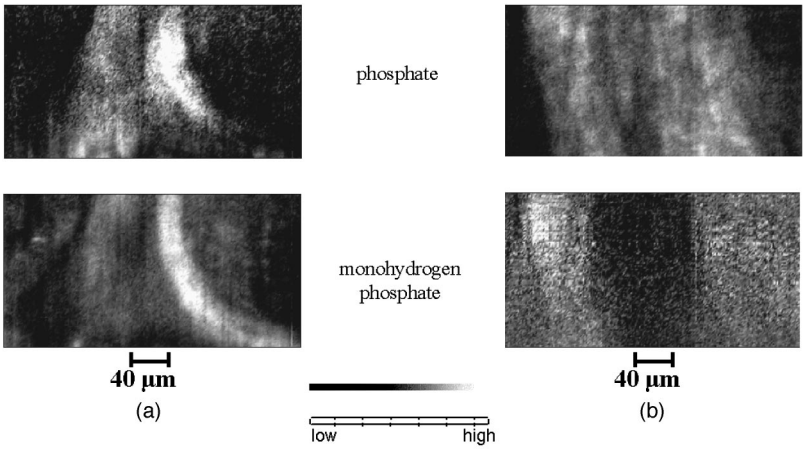

Fig. 8 Hyperspectral Raman images of phosphate and monohydrogen phosphate factors in a mature (a) and newly modeled (b) trabecular strut. Spatial resolution 2.8 μm, spectral resolution 8 cm^{-1}. Reprinted from Timlin et al., ''Spatial distribution of phosphate species in mature and newly generated mammalian bone by hyperspectral Raman imaging,'' *J. Biomed. Opt.* **4**(1), 28–34, copyright 1999, with permission from SPIE.

mercury–cadmium–telluride focal-plane array detector, eliminating the need for point mapping. Marcott and colleagues were able to collect 2.1×2.1 mm hyperspectral images of canine jawbone in approximately 5 min.[53] Recently, Mendelsohn and co-workers have compared parameters obtained from IR imaging experiments with those obtained from traditional IR microscopy experiments and have found them to be identical, thus validating the IR imaging technique.[54] Figure 9 shows an example of the spatially informative data that can be acquired using IR imaging.

Although vibrational imaging has so far been done mostly on a qualitative basis, this area of research holds great promise for future experiments. As the image acquisition speed increases, more and more ''real-time'' experiments can be

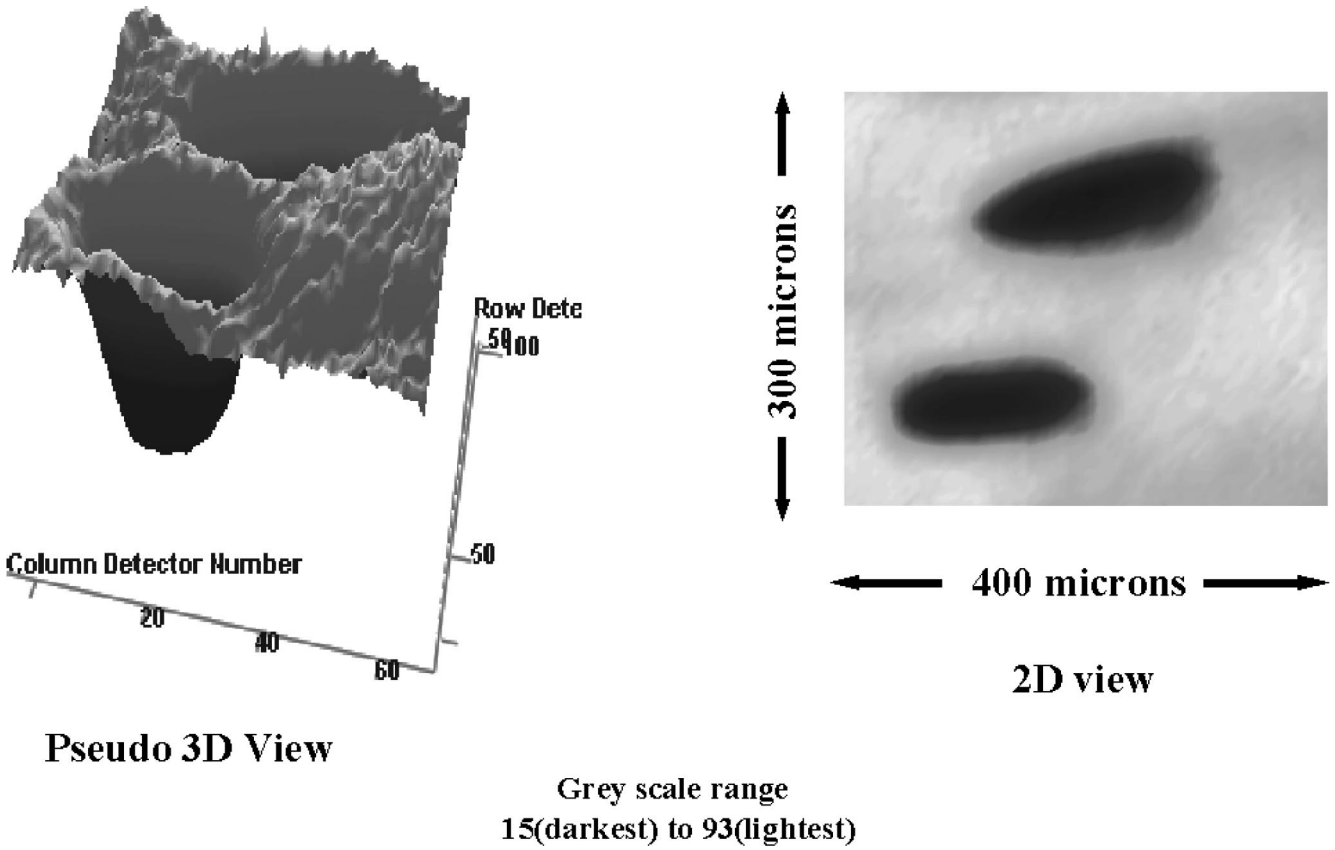

Fig. 9 Infrared spectroscopic image of the spatial distribution of apatitic mineral around two osteons from a human iliac crest biopsy. The image was constructed from the integrated area of the phosphate ν_1, ν_3 mode envelope between 950–1250 cm^{-1}. Scan time 4 min, spectral resolution 8 cm^{-1}, spatial resolution 10 μm. The authors thank Dr. E. Paschalis, Dr. A. Boskey, and Dr. R. Mendelsohn for providing this figure.

realized. Results comparable in quality and information to those gained from point spectroscopy measurements are not far away.

13 Conclusions

Both Raman spectroscopy and infrared spectroscopy are proving to be valuable tools for the study of mineralized tissues. The information that can be gained using these techniques is more comprehensive than that rendered by other methods, and the fact that the samples remain intact after examination opens up new possibilities for continuous or incremental experiments that would yield not only spatial but also temporal information.

The advent of vibrational imaging as applied to mineralized tissues is particularly exciting. This new method holds great promise for generating high definition chemical-state images that may prove invaluable in the study of mineralized tissue physiology and even in clinical diagnostics.

Acknowledgments

The authors would like to thank the Center for Biomedical Engineering Research at the University of Michigan Medical School and the NIH Cellular Biotechnology Training Program, Grant No. T32 GM08353, for financial support.

References

1. F. S. Kaplan, W. C. Hayes, T. M. Keaveny, A. Boskey, T. A. Einhorn, and J. P. Iannotti, ''Form and function of bone,'' in *Orthopaedic Basic Science*, S. R. Simon, Ed., pp. 127–184, American Academy of Orthopaedic Surgeons, Rosemont, IL (1994).
2. N. L. Pleshko, A. L. Boskey, and R. Mendelsohn, ''An FT-IR microscopic investigation of the effects of tissue preservation on bone,'' *Calcif. Tissue Int.* **51**, 72–77 (1992).
3. N. L. Pleshko, A. L. Boskey, and R. Mendelsohn, ''An infrared study of the interaction of polymethyl methacrylate with the protein and mineral components of bone,'' *J. Histochem. Cytochem.* **40**(9), 1413–1417 (1992).
4. I. Rehman, R. Smith, L. L. Hench, and W. Bonfield, ''FT-Raman spectroscopic analysis of natural bones and their comparison with bioactive glasses and hydroxyapatite,'' *Bioceramics* **7**, 79–84 (1994).
5. G. Penel, G. Leroy, and E. Bres, ''New preparation method of bone samples for Raman microspectrometry,'' *Appl. Spectrosc.* **52**(2), 312–313 (1998).
6. G. R. Sauer, W. B. Zunic, J. R. Durig, and R. E. Wuthier, ''Fourier transform Raman spectroscopy of synthetic and biological calcium phosphates,'' *Calcif. Tissue Int.* **54**, 414–420 (1994).
7. I. Rehman, R. Smith, L. L. Hench, and W. Bonfield, ''Structural evaluation of human and sheep bone and comparison with synthetic hydroxyapatite by FT-Raman spectroscopy,'' *J. Biomed. Mater. Res.* **29**, 1287–1294 (1995).
8. R. Smith and I. Rehman, ''Fourier transform Raman spectroscopic studies of human bone,'' *J. Mater. Sci.: Mater. Med.* **5**, 775–778 (1995).
9. B. Schrader, B. Dippel, I. Erb, S. Keller, T. Lochte, H. Schulz, E. Tatsch, and S. Wessel, ''NIR Raman spectroscopy in medicine and biology: Results and aspects,'' *J. Mol. Struct.* **480–481**, 21–32 (1999).
10. G. Penel, G. Leroy, C. Rey, and E. Bres, ''MicroRaman spectral study of the PO_4 and CO_3 vibrational modes in synthetic and biological apatites,'' *Calcif. Tissue Int.* **63**, 475–481 (1998).
11. A. Bertoluzza, C. Fagnano, A. Tinti, M. A. Morelli, M. R. Tosi, G. Maggi, and P. G. Marchetti, ''Raman and infrared spectroscopic study of the molecular characterization of the biocompatibility of prosthetic biominerals,'' *J. Raman Spectrosc.* **25**, 109–114 (1994).
12. B. Dippel, R. Mueller, A. Pingsmann, and B. Schrader, ''Composition, constitution, and interaction of bone with hydroxyapatite coatings determined by FT-Raman microscopy,'' *Biospectroscopy* **4**, 403–412 (1998).
13. C. J. de Grauw, J. D. de Bruijn, C. Otto, and J. Greve, ''Investigation of bone and calcium phosphate coatings and crystallinity determination using Raman microspectroscopy,'' *Cells Mater.* **6**(1–3), 57–62 (1996).
14. G. Penel, N. Leroy, P. van Landuyt, B. Flautre, P. Hardouin, J. Lemaitre, and G. Leroy, ''Raman microspectrometry studies of brushite cement: In vivo evolution in a sheep model,'' *Bone* **25**(2), 81S–84S (1999).
15. F. F. M. de Mul, M. H. J. Hottenhuis, P. Bouter, J. Greve, J. Arends, and J. J. ten Bosch, ''Micro-Raman line broadening in synthetic carbonated hydroxyapatite,'' *J. Dent. Res.* **65**(3), 437–440 (1986).
16. C. Rey, M. Shimizu, B. Collins, and M. J. Glimcher, ''Resolution-enhanced Fourier transform infrared spectroscopy study of the environment of phosphate ions in the early deposits of a solid phase of calcium phosphate in bone and enamel, and their evolution with age. II. Investigations in the υ_3 PO_4 domain,'' *Calcif. Tissue Int.* **49**, 383–388 (1991).
17. C. Rey, M. Shimizu, B. Collins, and M. J. Glimcher, ''Resolution-enhanced Fourier transform infrared spectroscopy study of the environment of phosphate ions in the early deposits of a solid phase of calcium phosphate in bone and enamel, and their evolution with age. I. Investigations in the υ_4 PO_4 domain,'' *Calcif. Tissue Int.* **46**, 384–394 (1990).
18. H. El. Feki, C. Rey, and M. Vignoles, ''Carbonate ions in apatites: Infrared investigations in the υ_4 CO_3 domain,'' *Calcif. Tissue Int.* **49**, 269–274 (1991).
19. C. Rey, V. Renugopalakrishnan, M. Shimizu, B. Collins, and M. J. Glimcher, ''A resolution-enhanced Fourier transform infrared spectroscopic study of the environment of the CO_3^{2-} ion in the mineral phase of enamel during its formation and maturation,'' *Calcif. Tissue Int.* **49**, 259–268 (1991).
20. C. Rey, B. Collins, T. Goehl, I. R. Dickson, and M. J. Glimcher, ''The carbonate ion environment in bone mineral: A resolution-enhanced Fourier transform infrared spectroscopy study,'' *Calcif. Tissue Int.* **45**, 157–164 (1989).
21. N. L. Pleshko, A. L. Boskey, and R. Mendelsohn, ''Novel infrared spectroscopic method for the determination of crystallinity of hydroxyapatite minerals,'' *Biophys. J.* **60**, 786–793 (1991).
22. N. P. Camacho, S. Rinnerthaler, E. P. Paschalis, R. Mendelsohn, A. L. Boskey, and P. Fratzl, ''Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy,'' *Bone* **25**(3), 287–293 (1999).
23. R. Mendelsohn, A. Hassankhani, E. DiCarlo, and A. Boskey, ''FT-IR microscopy of endochondral ossification at 20μ spatial resolution,'' *Calcif. Tissue Int.* **44**, 20–24 (1989).
24. A. L. Boskey, N. P. Camacho, R. Mendelsohn, S. B. Doty, and I. Binderman, ''FT-IR microscopic mappings of early mineralization in chick limb bud mesenchymal cell cultures,'' *Calcif. Tissue Int.* **51**, 443–448 (1992).
25. A. L. Boskey, N. Pleshko, S. B. Doty, and R. Mendelsohn, ''Applications of Fourier transform infrared (FT-IR) microscopy to the study of mineralization in bone and cartilage,'' *Cells Mater.* **2**(3), 209–220 (1992).
26. A. L. Boskey, S. Gadaleta, C. Gundberg, S. B. Doty, P. Ducy, and G. Karsenty, ''Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteocalcin,'' *Bone* **23**(3), 187–196 (1998).
27. S. Bohic, R. Rohanizadeh, S. Touchais, A. Godard, G. Daculsi, and D. Heymann, ''Leukemia inhibitory factor and oncostatin M influence the mineral phases formed in a murine heterotopic calcification model: A Fourier transform-infrared microspectroscopic study,'' *J. Bone Miner. Res.* **13**(10), 1619–1632 (1998).
28. A. Tsuda and J. Arends, ''Orientational micro-Raman spectroscopy on hydroxyapatite single crystals and human enamel crystallites,'' *J. Dent. Res.* **73**(11), 1703–1710 (1994).
29. H. Tsuda, J. Ruben, and J. Arends, ''Raman spectra of human dentin material,'' *Eur. J. Oral Sci.* **104**, 123–131 (1996).
30. M. J. Bottero, J. Yvon, and J. Vadot, ''Multimethod analysis of apatites in sound human tooth enamel,'' *Eur. J. Mineral.* **4**, 1347–1357 (1992).
31. M. G. Sowa and H. H. Mantsch, ''FT-IR photoacoustic depth profiling spectroscopy of enamel,'' *Calcif. Tissue Int.* **54**, 481–485 (1994).
32. M. G. Sowa and H. H. Mantsch, ''FT-IR step-scan photoacoustic

phase analysis and depth profiling of calcified tissue,'' *Appl. Spectrosc.* **48**(3), 316–319 (1994).
33. J. P. Cassella, R. Pereira, J. S. Khillan, D. J. Prockop, N. Garrington, and S. Y. Ali, ''An ultrastructural, microanalytical, and spectroscopic study of bone from a transgenic mouse with a COL1.A1 pro-alpha-1 mutation,'' *Bone* **15**(6), 611–619 (1994).
34. E. P. Paschalis, O. Jacenko, B. Olsen, R. Mendelsohn, and A. L. Boskey, ''Fourier transform infrared microspectroscopic analysis identifies alterations in mineral properties in bones from mice transgenic for type X collagen,'' *Bone* **19**(2), 151–156 (1996).
35. N. P. Camacho, C. M. Rimnac, R. A. Meyer, Jr., S. Doty, and A. L. Boskey, ''Effect of abnormal mineralization on the mechanical behavior of X-linked hypophosphatemic mice femora,'' *Bone* **17**(3), 271–278 (1995).
36. E. P. Paschalis, F. Betts, E. DiCarlo, R. Mendelsohn, and A. L. Boskey, ''FTIR microspectroscopic analysis of human iliac crest biopsies from untreated osteoporotic bone,'' *Calcif. Tissue Int.* **61**, 487–492 (1997).
37. R. Legros, N. Balmain, and G. Bonel, ''Age-related changes in mineral of rat and bovine cortical bone,'' *Calcif. Tissue Int.* **41**, 137–144 (1987).
38. C. Rey, V. Renugopalakrishnan, B. Collins, and M. J. Glimcher, ''Fourier transform infrared spectroscopic study of the carbonate ions in bone mineral during aging,'' *Calcif. Tissue Int.* **49**, 251–258 (1991).
39. J.-M. Very, R. Gibert, B. Guilhot, M. Debout, and C. Alexandre, ''Effect of aging on the amide group of bone matrix measured by FTIR spectrophotometry in adult subjects deceased as a result of violent death,'' *Calcif. Tissue Int.* **60**, 271–275 (1997).
40. H. G. M. Edwards, A. C. Williams, and D. W. Farwell, ''Paleodental studies using FT-Raman spectroscopy,'' *Biospectroscopy* **1**, 29–36 (1995).
41. M. T. Kirchner, H. G. M. Edwards, D. Lucy, and A. M. Pollard, ''Ancient and modern specimens of human teeth: A Fourier transform Raman spectroscopic study,'' *J. Raman Spectrosc.* **28**, 171–178 (1997).
42. A. Bertoluzza, P. Brasili, L. Castri, F. Facchini, C. Fagnano, and A. Tinti, ''Preliminary results in dating human skeletal remains by Raman spectroscopy,'' *J. Raman Spectrosc.* **28**, 185–188 (1997).
43. V. Michel, Ph. Ildefonse, and G. Morin, ''Assessment of archaeological bone and dentine preservation from Lazaret Cave (Middle Pleistocene) in France,'' *Palaeogeography, Palaeoclimatology, Palaeoecology* **126**, 109–119 (1996).
44. Y. Dauphin, ''Potential of the diffuse reflectance infrared Fourier transform (DRIFT) method in paleontological studies of bones,'' *Appl. Spectrosc.* **47**(1), 52–55 (1993).
45. E. Wentrup-Byrne, C. A. Armstrong, R. S. Armstrong, and B. M. Collins, ''Fourier transform Raman microscopic mapping of the molecular components in a human tooth,'' *J. Raman Spectrosc.* **28**, 151–158 (1997).
46. C. J. de Grauw, C. Otto, and J. Greve, ''Line-scan Raman microspectrometry for biological applications,'' *Appl. Spectrosc.* **51(11)**, 1607–1612 (1997).
47. D. M. Wieliczka, P. Spencer, and M. B. Kruger, ''Raman mapping of the dentin/adhesive interface,'' *Appl. Spectrosc.* **50**(12), 1500–1504 (1996).
48. J. A. Timlin, A. Carden, M. D. Morris, J. F. Bonadio, C. E. Hoffler, K. M. Kozloff, and S. A. Goldstein, ''Spatial distribution of phosphate species in mature and newly generated mammalian bone by hyperspectral Raman imaging,'' *J. Biomed. Opt.* **4**(1), 28–34 (1999).
49. J. A. Timlin, A. Carden, and M. D. Morris, ''Chemical microstructure of cortical bone probed by Raman transects,'' *Appl. Spectrosc.* **53**(11), 1429–1435 (1999).
50. A. Carden, J. A. Timlin, C. M. Edwards, M. D. Morris, C. E. Hoffler, K. M. Kozloff, and S. A. Goldstein, ''Raman imaging of bone mineral and matrix: composition and function,'' *Proc. SPIE* **3608**, 132–138 (1999).
51. E. P. Paschalis, F. Betts, E. DiCarlo, R. Mendelsohn, and A. L. Boskey, ''FTIR-microspectroscopic analysis of normal human cortical and trabecular bone,'' *Calcif. Tissue Int.* **61**, 480–486 (1997).
52. E. P. Paschalis, E. DiCarlo, F. Betts, P. Sherman, R. Mendelsohn, and A. L. Boskey, ''FTIR microspectroscopic analysis of human osteonal bone,'' *Calcif. Tissue Int.* **59**, 480–487 (1996).
53. C. Marcott, R. C. Reeder, E. P. Paschalis, D. N. Tatakis, A. L. Boskey, and R. Mendelsohn, ''Infrared microspectroscopic imaging of biomineralized tissues using a mercury-cadmium-telluride focal-plane array detector,'' *Cell Mol. Biol.* **44**(1), 109–115 (1998).
54. R. Mendelsohn, E. P. Paschalis, and A. L. Boskey, ''Infrared spectroscopy, microscopy, and microscopic imaging of mineralizing tissues: spectra-structure correlations from human iliac crest biopsies,'' *J. Biomed. Opt.* **4**(1), 14–21 (1999).