Infrared Assessment of Bone Quality

A Review

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Abstract

Background Bone strength depends on both bone quantity and quality. The former is routinely estimated in clinical settings through bone mineral density measurements but not the latter. Bone quality encompasses the structural and material properties of bone. Although its importance is appreciated, its contribution in determining bone strength has been difficult to precisely quantify partly because it is multifactorial and requires investigation of all bone hierarchical levels. Fourier transform infrared spectroscopy provides one way to explore these levels.

Questions/purposes The purposes of our review were to (1) provide a brief overview of Fourier transform infrared spectroscopy as a way to establish bone quality, (2) review the major bone material parameters determined from Fourier transform infrared spectroscopy, and (3) review the role of Fourier transform infrared microspectroscopic analysis in establishing bone quality.

Methods We used the ISI Web of Knowledge database initially to identify articles containing the Boolean term "infrared" AND "bone." We then focused on articles on infrared spectroscopy in bone-related journals.

Results Infrared spectroscopy provides information on bone material properties. Their microspectroscopic versions allow one to establish these properties as a function of anatomic location, mineralization extent, and bone metabolic activity. It provides answers pertaining to the contribution of mineral to matrix ratio, mineral maturity, mineral carbonate substitution, and collagen crosslinks to bone strength. Alterations of bone material properties have been identified in disease (especially osteoporosis) not attainable by other techniques.

Conclusions Infrared spectroscopic analysis is a powerful tool for establishing the important material properties contributing to bone strength and thus has helped better understand changes in fragile bone.

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Introduction

Loss of bone mass, measured clinically as change in bone mineral density (BMD), is considered an important risk factor for bone fragility. However, it is not the sole predictor of whether an individual will experience a fracture [8, 43]. Moreover, considerable overlap in BMD exists between populations that do and do not develop fractures [14, 41, 45]. For a given bone mass, an individual's risk to fracture increases with age [32]. Additionally, numerous reports document mechanical variables directly related to fracture risk are either independent [33] or not solely dependent on bone mass itself [34, 35, 44, 50, 53]. In a recent report analyzing iliac crest biopsies from 54 women (32 with fractures, 22 without) who had lower (compared

with normal) spine but not hip BMDs, cortical and cancellous collagen maturity strongly correlated with overall fracture incidence (increased with increased fracture risk) [27], emphasizing the contribution of collagen quality in determining bone strength.

It is becoming evident, in addition to BMD, bone quality should also be considered when assessing bone strength and fracture risk. Bone quality is a broad term encompassing factors affecting the structural and material properties of bone (Fig. 1), both of which mainly depend on bone turnover. Notable potential exceptions (at least as far as material properties are concerned) are cases in which factors directly affecting the physical chemistry of mineral crystallites are involved. Such a case would be bisphosphonates as they adsorb onto the apatitic surfaces, changing the surface properties, and affect the rate of mineral growth and dissolution [29–51]. Other examples would be strontium [40, 71] as it incorporates into the apatitic mineral, changing its dissolution characteristics and crystallite size and shape, and fluoride [21, 23, 69, 70] as it incorporates into the apatitic mineral crystallites, making them larger, and greatly reduces the dissolution rate of these crystallites. As far as collagen properties are concerned, an example would be homocysteine [1, 75] as it interferes with collagen enzymatic posttranslation modifications that occur after it has been synthesized and excreted by the osteoblast.

One of the obstacles to be circumvented when assessing mineral and matrix tissue properties is tissue heterogeneity at the microscopic level. Bone surfaces may be undergoing formation and/or resorption or they may be inactive. These processes, which can be visualized microscopically, occur throughout life in both cortical and trabecular bone [18]. Bone remodeling is a surface phenomenon and in humans occurs on periosteal, endosteal, Haversian canal, and trabecular surfaces [9, 10, 18, 64]. The rate of cortical bone remodeling, as high as 50% per year in the midshaft of the femur during the first 2 years of life, eventually declines to a rate of 2% to 5% per year in the elderly. Rates of remodeling in trabecular bone are proportionately higher throughout life and may normally be five to 10 times higher than cortical bone remodeling rates in the adult [18]. This information is critical when evaluating bone at the microscopic level; thus, variability in tissue age should be accounted for.

Fourier transform infrared (FTIR) spectroscopy provides one way to explore bone quality at multiple bone hierarchical levels. The purposes of our review were to (1) provide a brief overview of FTIR spectroscopy as a way to establish bone quality and explore tissue variability, (2) review the major bone material parameters determined from FTIR spectroscopy, and (3) review the role of FTIR microspectroscopic analysis in establishing bone quality.

Search Strategy and Criteria

We used the ISI Web of Knowledge database initially to identify articles containing the Boolean term "infrared" AND "bone." This resulted in 1640 hits. To narrow the

Fig. 1 A flow diagram shows factors contributing to bone quality and bone strength.
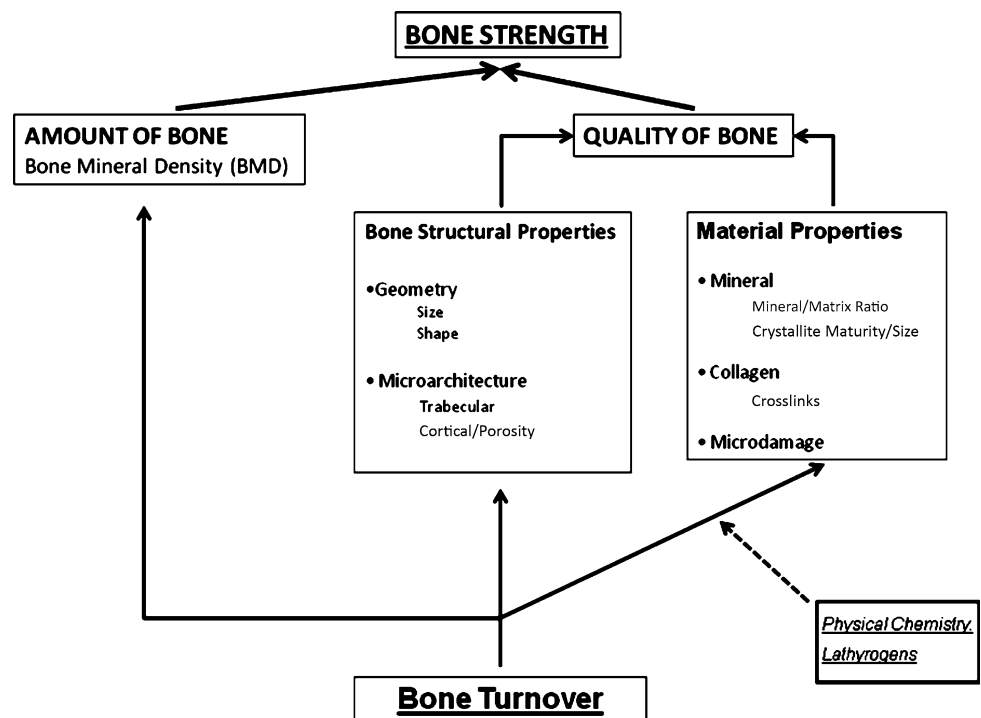
number of articles identified, we used additional terms such as "aging," "osteoporosis," "fracture," "mineral," "carbonate," and "collagen," which resulted in 99, 49, 80, 711, 216, and 229 hits, respectively. The searches were then focused primarily on infrared spectroscopy articles published in bone-related journals and bone studies published in peer-reviewed chemical spectroscopy and biomedical optical journals.

FTIR Spectroscopy

All chemical bonds undergo vibrations that encompass a variety of motions of the atomic groupings within the molecular framework. These include bond stretching, in- and out-of-plane angle bending, wagging (change in angle between a bond and a plane), and twisting (change in angle between two planes). If a particular motion is accompanied by a dipole moment change, when illuminated with infrared radiation, it will absorb at a specific infrared frequency, characteristic of the overall configuration of the atoms and representative of specific functional groups. Moreover, through detailed analysis of the absorption spectra, information may be derived on subtle interactions with the surrounding groups of a molecule. FTIR spectra provide information on all bone tissue components (Fig. 2). The protein and mineral constituents produce intense, structure-sensitive infrared modes. The peaks of interest have been appropriately marked. Information is available from both the mineral (carbonate substituting in the apatite lattice and phosphate from the apatite itself) and collagen (Amide I, II, and III; the first results from peptide bond C=O stretch, the second results from mixed C–N stretch and N–H in-plane bend, and the third also results from mixed C–N

Fig. 2 A typical FTIR spectrum of bone powder is shown. Information on both the inorganic and organic constituents of the bone composite is readily discernible. FTIR = Fourier transform infrared.

stretch and N–H in-plane bend with additional contributions from C–C$_\alpha$ stretch).

FTIR Spectroscopy Major Bone Material Parameters

The most frequently reported parameters of FTIR spectroscopy pertaining to bone material properties are (1) mineral to matrix ratio, (2) mineral maturity/crystallinity, and (3) collagen maturity (expressed as the ratio of two of the major Type I bone collagen crosslinks).

Mineral to Matrix Ratio

The integrated area under an infrared band is directly proportional to the amount of species that generates the band. As a result, the ratio of the integrated phosphate and any of the amide bands (usually Amide I) is representative of the amount of mineral normalized to the amount of collagen present. It is a measure of BMD and correlates with ash weight measurements [6]. Nevertheless, caution should be exercised when the results are considered and compared with conventional BMD parameters such as BMD and BMD distribution (BMDD) because this infrared spectra parameter provides information on the amount of mineral per volume analyzed per amount of collagen present, whereas BMD and BMDD express amount of mineral per volume. It is biologically relevant because it accounts for both the main constituents of bone but suffers from the fact that it is a ratio (for example, an unaltered ratio may be the result of the fact that there is no change in either of the ratio components or a proportionate one in both). It is most informative when combined with results of a technique such as quantitative backscattered electron imaging, which provides information on the amount of mineral distribution at the microscopic level [72]. On the other hand, it may provide a more relevant description of whether bone tissue is either hyper- or hypomineralized because it encompasses both major constituents of bone (mineral and collagen), and because bone is considered a composite material, its mechanical performance depends on the quantity and quality of both.
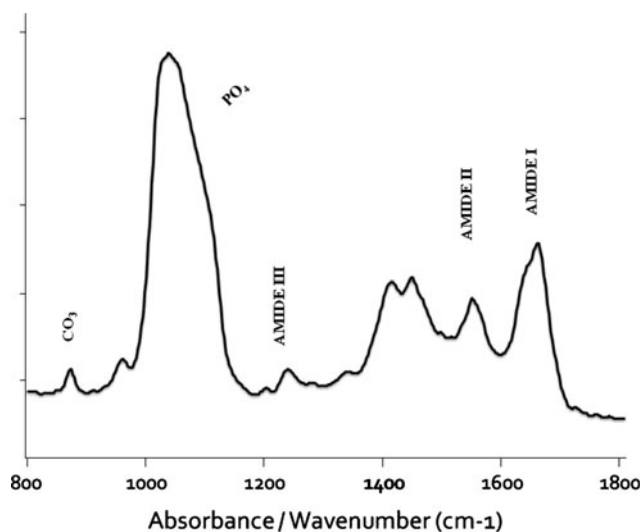
Mineral Maturity/Crystallinity

FTIR spectroscopy has been extensively used in the analysis of bone mineral [3, 63, 65, 74]. Through spectroscopic and mathematical analysis of the phosphate band by means of techniques such as deconvolution, second-derivative spectroscopy, and curve fitting, spectral regions (underlying peaks) have been identified and correlated with the various chemical environments present in biologic apatites,

enabling the monitoring of the calcium phosphate crystal maturity (ionic substitutions in the poorly crystalline apatite lattice, stoichiometry) [2, 3, 66–68, 73]. This mineral characteristic changes as a function of tissue age [54] as a result of the dynamic physical chemistry status of the crystals bathing in biologic fluids, resulting in variable mineral maturity within the same specimen. For example, not only carbonate content but also the type of substitution (Type A represents carbonate in the hydroxyl position of apatite, Type B represents carbonate in the phosphate, and labile represents loosely adsorbed carbonate on the crystal surfaces) changes as a function of tissue age [54], influencing the solubility and crystallinity (shape and size) of apatite crystallites.

Based on physical chemical studies [20, 54], it is known, as bone mineral crystals age, so does the crystallinity of the apatite mineral. As a result, efforts have been made to extrapolate from the direct measure of FTIR spectroscopy, which is mineral maturity, to mineral crystallinity [24, 54]. Nevertheless, caution should be exercised in that the synthetic mineral crystallites used in the in vitro experiments are not subjected to any ordering and/or orientation, unlike bone mineral crystallites. This may be the underlying cause for the paradox that the same spectroscopic parameter in experiments involving powders correlates well with the c-crystallographic axis (crystallite length, determined by

xray diffraction analysis), whereas in tissue sections, it correlates with crystallite thickness (determined by small-angle xray spectroscopic analysis) [12, 24, 54]. The important contribution of mineral maturity/crystallinity in determining bone strength may be appreciated in the case of fluoride-treated osteoporotic bone. In that instance, despite gains in BMD, bone eventually becomes fragile because of the crystallites' increased maturity/crystallinity due to the physical chemical effect of fluoride on biologic apatites [21, 23, 69, 70].

The v_1, v_3 PO_4^{3-} infrared band in mineralized tissues is a composite one, reflecting the presence of nanocrystalline mineral; the band consists of underlying peaks, each of which is representative of a specific chemical environment. The major underlying bands (as determined through second-derivative spectroscopy and curve-fitting routines) are summarized (Table 1) [54]. The relative distribution of these bands varies as a result of the dynamic nature of poorly crystalline apatitic crystals.

Collagen Maturity and Collagen Crosslinks

The protein Amide I (peptide bond C=O stretch) and Amide II (mixed C–N stretch and N–H in-plane bend) modes near 1650 and 1550 wavenumbers (cm^{-1}) undergo

Table 1. Major components of the v_1, v_3 PO_4^{3-} and Amide I infrared bands [36, 57]

Band	Underlying peak position (cm^{-1})	Assignment
v_1, v_3 PO_4^{3-}	960	v_1 PO_4^{3-}
	996	PO_4^{3-} in apatitic environment
	1020	Persistence of vacancies; nonstoichiometric apatites containing HPO_4^{2-} and/or CO_3^{2-}
	1032	PO_4^{3-} in stoichiometric apatites
	1044	Type B carbonate apatites; hydroxyapatite
	1056	Bands corresponding to the T_2 vibrational modes of apatite
	1075	Bands corresponding to the T_2 vibrational modes of apatite
	1092	Stoichiometric apatites
	1109	Poorly crystalline apatites
	1123	HPO_4^{2-}
	1143	HPO_4^{2-} containing apatites, mostly biological
Amide I	1625	Beta sheets
	1634	Beta sheets
	1646	Unordered (random)
	1654	Alpha helix
	1660	3_{10} helix; pyridinoline collagen crosslinks
	1674	Beta structure
	1683	Beta turn
	1690	Beta turn; divalent collagen crosslinks
	1694	Beta turn

frequency and intensity changes as a result of changes in protein secondary structure [25]. As in the case of mineral analysis, information on protein structure is extracted from broad spectral bands consisting of component peaks resulting from the Amide I modes of various secondary structures by using a spectral resolution technique such as Fourier self-deconvolution, second-derivative spectroscopy, and difference FTIR spectroscopy [15, 25, 36–39, 76]. The major underlying bands and their assignments are summarized (Table 1). Fairly recently, a method was developed enabling the spectral analysis of the Amide I band to isolate underlying peaks corresponding to two of the major Type I bone collagen crosslinks, namely pyridinoline (pyr) and divalent ones [61].

FTIR Microspectroscopy and Imaging

Despite the fact that detailed information on mineral maturity and protein secondary structure is obtainable using FTIR analysis, homogenized bone tissue is a prerequisite, making it impossible to correlate the monitored bone material properties with tissue age and bone metabolic activity, thus greatly dependent on bone turnover.

In the late 1980s, the combination of an optical microscope with an infrared spectrometer offered the opportunity to examine thin bone tissues with a spatial resolution of approximately 20 μm [46]. This, for the first time as far as FTIR analyses were concerned, allowed the selection of anatomic areas to be investigated based on parallel histologically stained sections and enabled the correlation of the spectroscopic results with bone surface metabolic activity (tissue age) [46, 57, 61]. This work was later replicated and expanded by others [11, 16, 19, 30, 31, 47, 48], resulting in a wealth of new information about the mineral component of bone as a function of cellular activity, tissue age, disease, and therapeutic intervention.

Another achievement was the development of spectroscopic parameters corresponding to two of the major mineralizing Type I collagen crosslinks (pyr and dehydrodihydroxynorleucine [DHLNL]) in thin, histologically stained bone sections [61]. This allowed the establishment of the variation in their spatial distribution as a function of anatomic location, cellular activity, and tissue age [61]. As a consequence, it is feasible to describe differences between healthy and diseased bone independent of bone turnover status.

One major drawback of FTIR microspectroscopic analysis using a single infrared detector element (area of analysis of 10 × 10 μm) is the time required for spectral acquisition and further spectral processing (so as to calculate the relevant parameters pertaining to mineral crystallinity and collagen crosslinks) over a large area (so as to obtain statistically meaningful results) of a single biopsy, rendering the efficient analysis of biopsies virtually impossible. This limitation was overcome through the development of infrared microspectroscopic imaging. The approach required the integration of an infrared focal-plane array detector and a FTIR microscope [42]. The advantage of this technique lies in the fact that the spectral acquisition and processing time is shortened by at least 1000-fold compared with conventional FTIR microspectroscopy. Using a step-scanning FTIR spectrometer with an mercury cadmium telluride array detector placed at the image focal plane of the FTIR microscope enables areas of approximately 400 × 400 μm² to be analyzed in less than 3 to 4 minutes with a spatial resolution of approximately 6.3 μm [42]. Instead of a single FTIR spectrum, 4096 spectra are collected simultaneously in a 64 × 64 array configuration. Through integration of the appropriate bands, high-fidelity chemical images providing information on the spatial distribution of mineral, collagen, and polymethylmethacrylate (embedding medium) may be portrayed (in all images, blue = minimum, red = maximum). Through further spectral and mathematical processing and resolution of the raw bands into their constituent underlying peaks, the spatial distribution of the mineral crystallite maturity/crystallinity and pyr/divalent collagen crosslinks may be calculated and presented (Fig. 3) [28, 57, 59–61]. To date, it has been successfully applied in the analysis of cell cultures and bones from animal models and humans [5, 28].

An Example of Important Findings Based on FTIR Analysis of Bone

Infrared analysis of bone tissue has proven a valuable tool in the field of osteoporosis and other conditions affecting bone quality such as osteopetrosis, osteomalacia, renal osteodystrophy, and osteogenesis imperfecta. Its use (and in particular the fact that it allows for anatomic area selection at the microscopic level) allowed for the detection of differences between normal and osteoporotic human bone both as far as mineral maturity/crystallinity (invariably higher in osteoporotic bone) and collagen crosslink ratio (invariably higher in osteoporotic patients at equivalent anatomic locations) are concerned [4, 59, 60]. These findings, especially the collagen crosslink ratio, strongly suggest factors other than bone turnover alone are partly responsible for the observed changes in material properties of osteoporotic bone, contributing to the clinically manifested fragility. For example, the changes in the collagen crosslink ratio observed in osteoporotic patients compared with normal humans would be consistent with the presence of elevated blood plasma homocysteine levels [1, 59, 60],
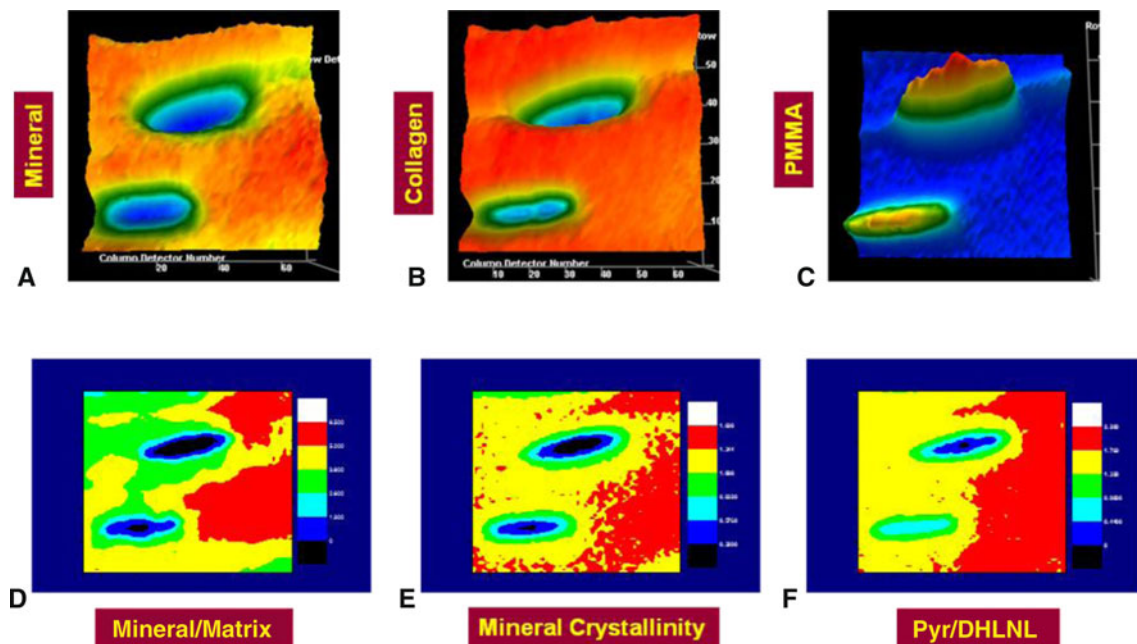
Fig. 3A–F An example of FTIR analysis of a thin section from a normal human iliac crest biopsy (cortical bone) with two evident osteons is shown. In all images, red corresponds to the highest and blue to the lowest values. Spatial distribution of the amount of (**A**) mineral, (**B**) collagen, and (**C**) polymethylmethacrylate (PMMA; biopsy embedding material) is provided. Additionally, the spatial distribution of (**D**) mineral to matrix ratio, (**E**) mineral crystallite maturity/crystallinity, and (**F**) collagen crosslink ratio (pyr/DHLNL) may be calculated from the raw spectral images. FTIR = Fourier transform infrared.

which has been recently proposed as an additional risk factor in osteoporosis.

Discussion

Bone strength depends on both bone quantity and quality. Bone quality encompasses the structural and material properties of bone. Although its importance is appreciated, its contribution in determining bone strength has been difficult to precisely quantify partly because it is multifactorial and requires investigation of all bone hierarchical levels. FTIR spectroscopy provides one way to explore bone quality at multiple bone hierarchical levels. In this review, we (1) provided a brief overview of FTIR spectroscopy as a way to establish bone quality and explore tissue variability, (2) reviewed the major bone material parameters determined from FTIR spectroscopy, and (3) reviewed the role of FTIR microspectroscopic analysis in establishing bone quality.

We bring to the reader's attention the following limitations to our review and the literature. Only a fraction of the available papers and specifically those in readily accessible peer-reviewed medical and chemical spectroscopy and biomedical optics journals have been cited. Details of the relevant spectroscopic theory and instrumentation have not been included. The interested reader can find them in advanced analytical chemistry textbooks and specialized books on infrared spectroscopy and its applications. A point the reader should bear in mind is that most studies involving FTIR microspectroscopic analysis of bone employ thin tissue sections. Since there are always microvariations in thickness even within the same section, it is unreliable to report absolute amounts as some of the referenced studies have done. The most commonly used way around this limitation is to report ratios rather than absolute amounts, thus rendering these thickness microvariations unimportant.

Both FTIR microspectroscopic and imaging analyses provide a wealth of otherwise unattainable information, yet we believe neither is well suited as a routine clinical screening tool because a bone biopsy is required. On the other hand, both are powerful research tools, providing unique insights into the pathophysiology of musculoskeletal diseases such as osteoporosis, osteogenesis imperfecta, Paget's disease, osteomalacia, osteopetrosis, osteosclerosis, and so on [28]. They are particularly useful in the investigation of exceptional clinical cases such as fracturing patients whose "classic" clinical risk indicators such as BMD and biochemical markers are normal [60]. The obtained bone material properties complement ones obtained through histology, histomorphometry, biochemical markers, blood analysis, and BMD measurements, thus offering unique information on the mechanisms that result

in healthy and diseased bone. They have also proven useful in evaluating various therapeutic protocols, providing insight into the mechanisms by which the various drugs work and therefore assisting in the design of new and more targeted ones [7, 13, 17, 22, 26, 49, 52, 55, 56, 58, 62, 71].

One of the unique capabilities of FTIR microspectroscopy and imaging is the potential to describe the spatial variation of pyr and DHLNL collagen crosslinks in mineralized thin tissue sections. These are only two of the major collagen crosslinks, and as a result only a partial understanding of the spatial and temporal distribution of collagen properties and their bearing on bone strength has been gained. In the future, spectral and mathematical methods should be developed to derive spectroscopic parameters that describe all of the known collagen crosslinks because they are important both in the mineralization initiation cascade of events and in determining bone strength.

In conclusion, infrared spectroscopic analysis is a powerful tool for establishing the important material properties contributing to bone strength and thus has helped better understand changes in fragile bone.

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