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Fourier transform infrared analysis and bone

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Why we need bone quality

Loss of bone mass, measured clinically as change in bone mineral density (BMD), is considered an important risk factor for bone fragility. However, BMD is not the sole predictor of whether an individual will experience a fracture [1, 2], and there is considerable overlap in BMD between populations that do and do not develop fractures [3–5]. It has been demonstrated that for a given bone mass, an individual's risk to fracture increases with age [6]. Consistent with these findings, numerous investigators have shown that mechanical variables directly related to fracture risk are either independent [7] or not totally accounted for bone mass itself [8–12]. Epidemiological evidence also shows considerable overlap of bone density values between fracture and non-fracture groups suggesting that low bone quantity alone is an insufficient cause of fragility fractures [13–15]. It is becoming evident then, that in addition to BMD, bone quality should also be considered when assessing bone strength and fracture risk. Bone quality is a broad term encompassing a plethora of factors such as geometry and bone mass distribution, trabecular bone microarchitecture, microdamage, increased remodeling activity, along with genetics, body size, environmental factors, and changes in bone mineral and matrix tissue properties [4, 5].

One of the obstacles to be circumvented when assessing mineral and matrix tissue properties is tissue heterogeneity at the microscopic level. In normal humans, cortical bone constitutes approximately 80% of the human skeletal mass and trabecular bone approximately 20% [16]. Bone surfaces may be undergoing formation or resorption, or they may be inactive. These processes, which can be visualized microscopically, occur throughout life in both cortical and trabecular bone [16]. Bone remodeling is a surface phenomenon and in humans occurs on periosteal, endosteal, Haversian canal, and trabecular surfaces [16–19]. The rate of cortical bone remodeling, which may be as high as 50% per year in the mid-shaft of the femur during the first 2 years of life, eventually declines to a rate of 2–5% per year in the elderly. Rates of remodeling in trabecular bone are proportionately higher throughout life and may normally be five to ten times higher than cortical bone remodeling rates in the adult [16]. This information is critical when evaluating bone at the microscopic level; thus, tissue age should be considered as well.

Fourier transform infrared spectroscopy

Molecular bonds are not stationary, but rather undergo motion such as twisting, bending, rotation, and vibration. When irradiated with infrared radiation, these vibrational motions absorb at specific wavelengths, characteristic of the overall configuration of the atoms, and representative of specific functional groups. Moreover, through detailed analysis of the absorption wavelengths, information may be deduced on the subtle interactions with the surrounding

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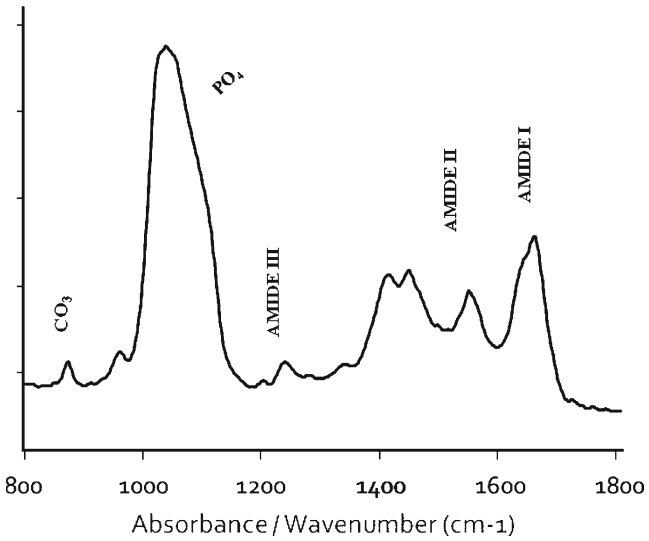
moieties of a molecule. Fourier transform infrared spectroscopy (FTIR) spectra provide information on all tissue components. The protein and mineral constituents produce intense, structure sensitive IR modes. Below is a typical FTIR spectrum of bone powder.



The characteristic peaks have been appropriately marked. As may be seen, information 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 is due to the carbonyl, the second due to the −NH groups, and the third due to both groups present in organic moieties) components is accessible.

FTIR major outcomes

The most frequently reported outcomes of FTIR spectroscopy are (1) mineral to matrix ratio, (2) mineral maturity, and (3) collagen maturity, and specifically the ratio of two of the major type I bone collagen cross-links.

Mineral to matrix ratio

The area under the infrared band is directly proportional to the amount of species that generates the respective band. As a result, the ratio of the integrated phosphate and any of the amide bands (usually amide I) is proportional to the amount of mineral corrected for the amount of collagen present. This is a measure of bone mineral density and has been shown to correlate with ash weight measurements [20]. Nevertheless, caution should be exercised when the results are considered and compared with conventional bone mineral density parameters such as BMD and bone mineral density distribution (BMDD) as the FTIR parameter expresses amount of mineral per volume analyzed per amount of collagen present, while BMD and BMDD express amount of mineral per volume.

Mineral maturity

IR spectroscopy has been extensively utilized in the analysis of bone mineral [21–24]. Spectroscopic and mathematical analysis of the phosphate band by means of techniques such as deconvolution, second derivative spectroscopy, and curvefitting, spectral regions (underlying peaks) were identified and correlated with the various chemical environments present in biological apatites, enabling the monitoring of the calcium phosphate crystal maturity (ionic substitutions in the poorly crystalline apatite lattice, stoichiometry) [24–29]. This mineral characteristic changes as a function of tissue age [30]. This is the result of the dynamic physical chemistry status of the crystals that are bathed in biological fluids. As a result of this dynamic situation, the ionic substitutions and thus the stoichiometry and maturity of the mineral crystallites are variable. For example, not only carbonate content but also the type of substitution (type A denotes carbonate in the hydroxyl position of apatite, type B carbonate in the phosphate, and labile signifies loosely adsorbed carbonate on the crystal surfaces) changes as a matter of changing maturity and tissue age [30]. The extent and type of carbonate substitution is known to affect the solubility and crystallinity of apatite crystallites. Based on solution physical chemical studies, it is known that as maturity changes, so does the crystallinity of the apatitic phase. Thus, efforts have been made to extrapolate from the direct measure of FTIR, which is mineral maturity, to mineral crystallinity [30, 31]. Although this is a useful extrapolated outcome, caution should be exercised in that the synthetic mineral crystallites employed in the in vitro experiments are not subjected to any ordering and/or orientation, unlike the case of biological mineral. 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 X-ray diffraction analysis), while in tissue sections, it correlates with crystallite thickness (as determined by small-angle X-ray spectroscopic analysis) [30–32].

Collagen maturity and collagen cross-links

The protein amide I (peptide bond C═O stretch) and amide II (mixed C–N stretch and N–H in-plane bend) modes near 1,650 and 1,550 wavenumbers (cm^{-1}) undergo frequency and intensity changes as a result of changes in protein secondary structure. The amide I band is especially sensitive to secondary structure [33]. In such studies,

information on protein structures is extracted from broad envelopes consisting of component bands arising from the amide I modes of various secondary structures by applying a technique of resolution enhancement such as Fourier self-deconvolution, second derivative spectroscopy, and difference FTIR [33–39]. Fairly recently, a method was developed that enables through the examination of the amide I and II bands to derive parameters corresponding to two of the major type I bone collagen cross-links, namely pyridinoline (pyr) and divalent ones [40].

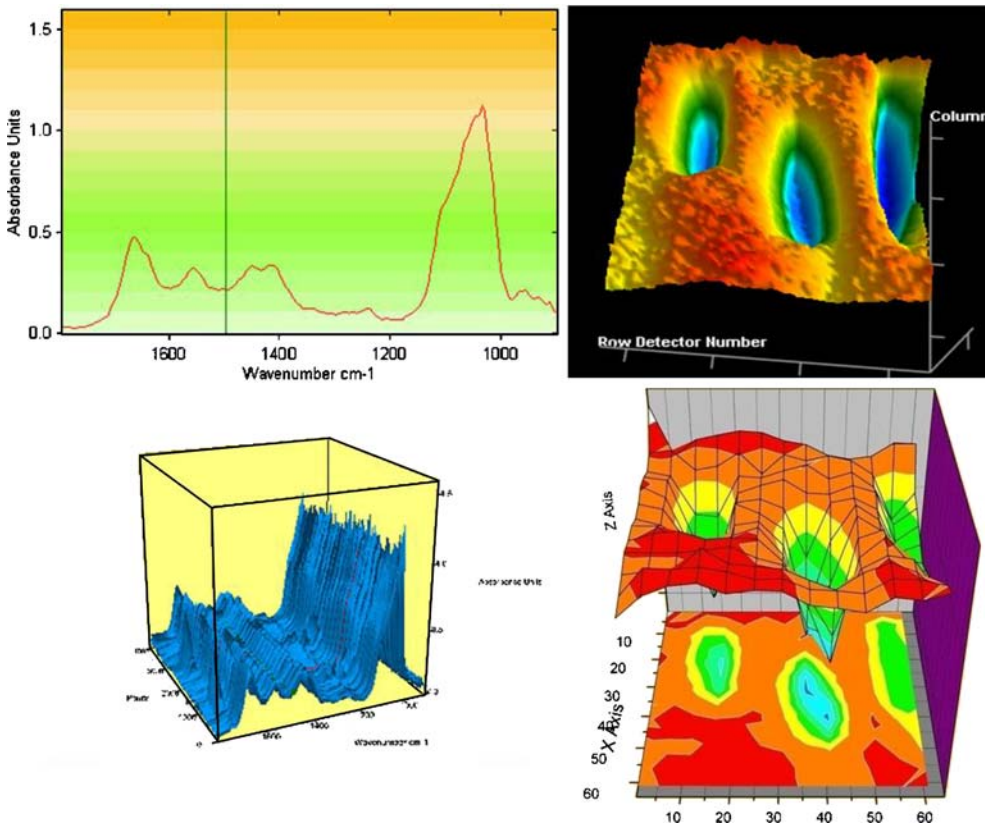
Fourier transform infrared microspectroscopy

Although detailed information on mineral maturity and protein secondary structure was obtainable utilizing these techniques, homogenized bone tissue and/or proteins in solution had to be used, making it impossible to correlate the outcomes with tissue age.

bone surface metabolic activity (tissue age) [30, 40, 41]. This work was later replicated and expanded by others [42–48], 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.

A major breakthrough was the development of spectroscopic parameters that enabled for the first time the monitoring of two of the major collagen cross-links (pyr and deH-DHLN) in thin, histologically stained bone sections, allowing the monitoring of the variation in their spatial distribution as a function of anatomical location, cellular activity, and tissue age [40].

As informative as it may be, FTIR microspectroscopic analysis utilizing a single infrared detector is time consuming, as analysis of a single section can easily last for 2–3 days. The recently available combination of an infrared focal-plane array detector and a FTIR microscope is a powerful one for obtaining spectroscopic images with unprecedented image fidelity [49, 50].



In the early 1990s, the coupling of an optical microscope with an infrared spectrometer offered the unique opportunity of studying thin bone tissues with a spatial resolution of ~10 μm and to select the anatomical areas to be analyzed based on parallel histologically stained sections, thus enabling the correlation of the spectroscopic result with

The above figure is an example of FTIRI analysis of a thin section from a human iliac crest biopsy (cortical bone), with three visible osteons. Instead of a single FTIR spectrum (upper left), 4096 ones are collected simultaneously (bottom left) in a 64×64 array configuration. Through integration of the amide I band, the spatial distribution of collagen may be

rendered (upper right image; blue=minimum, red=maximum). Through further spectral and mathematical manipulation of the raw spectra, the spatial distribution of the pyr/divalent collagen cross-links may be described (lower right image; blue=minimum, red=maximum) [40].

The advantage of this technique lies in the fact that the spectra acquisition and processing time is shortened by at least 1,000-fold compared to conventional FTIR microspectroscopy. Using a step-scanning FTIR spectrometer with a Mercury Cadmium Telluride array detector placed at the image focal plane of the FTIR microscope enables areas ~$400 \times 400\,\mu m^2$ to be collected in less than 3-4 min with a spatial resolution of ~6.3 μm. To date, it has been successfully applied in the analysis of cell cultures and bones from animal models and humans [51].

Future outlook

Since their introduction, both FTIR microspectroscopic and imaging analyses have been attempted to be established as diagnostic tools in the clinical setting. Although they both provide a wealth of otherwise unattainable information, it is the author's opinion that neither is well suited to be employed as a mass-screening tool, for the simple reason that it is an invasive technique as a bone biopsy is required. On the other hand, it is ideally suited for exceptional clinical cases such as fracturing patients whose "classical" risk indicators such as BMD and biochemical markers are normal [52].

On the other hand, they have proven to be powerful research tools, affording unique insights into the pathophysiology of musculoskeletal diseases such as osteoporosis, osteogenesis imperfecta, Paget's disease, osteomalacia, osteopetrosis, osteosclerosis, etc. The outcomes complement ones obtained through analyses such as histology, histomorphometry, biochemical markers, blood analysis, and BMD measurements, thus offering detailed information on the mechanisms that result in healthy and diseased bone. They have also proven useful in evaluating various therapeutic protocols, thus assisting in the design of more targeted ones.

One of the major advantages of FTIR microspectroscopy and imaging is that it can describe the spatial variation of pyr and deH-DHLNL collagen cross-links in mineralized thin tissue sections. These are only two of the major collagen cross-links, and as a result, only a partial understanding of the spatial and temporal distribution of collagen properties has been achieved. In the future, spectral and mathematical methods should be combined so as to derive spectroscopic parameters that describe all of the known collagen cross-links, as they are important both in the mineralization initiation cascade of events and in determining bone strength.

The main outcomes of Infrared microspectroscopic analysis correlate well with bone strength but are not the sole determinants. Moreover, a review of the literature reveals that the variation in the material and structural properties of bone is in the 1–10 μm range. It is necessary then in the future to combine infrared microspectroscopic analysis with other techniques capable of analyzing thin bone tissue sections with similar spatial resolution such as quantitative backscatter electron imaging (providing information on the bone mineral density distribution at the micrometer level), small-angle X-ray scattering (providing precise information on the mineral crystallite size, shape, and alignment to the collagen fibers), and nanoindentation (providing information on the bone mechanical properties at discrete anatomical location with a spatial resolution ~1 μm) at carefully selected (based on histology/histomorphometry to include cellular activity as a selection criterion) identical anatomical locations so that the contribution of each outcome to bone strength may be calculated [53].

In conclusion, FTIR microspectroscopy and imaging have proven to be powerful tools in the establishment of parameters contributing to bone quality and thus bone strength.

Conflicts of interest None

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