Mini-review

FTIR microspectroscopic analysis: future perspectives

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Summary

It is widely accepted that bone strength depends on both its structural and material properties. The latter, although important, are difficult to establish until fairly recently. The new generation of infrared microspectroscopic and imaging instruments offers a unique tool in determining the material properties of bone as they allow the study of thin tissue sections and the determination of important parameters such as mineral maturity/crystallinity and collagen cross-links ratio in a spatially resolved manner thus enabling the correlation with bone turnover. Despite the fact that the utility of such techniques as mass screening tools is currently debated since a biopsy is required, studies employing this technology have advanced our knowledge of the underlying mechanism of bone disease and the course of action of various therapeutic protocols.

KEY WORDS: infrared spectroscopy, bone quality, osteoporosis, bone strength, mineral maturity, collagen cross-links.

Bone

Bone is a composite material, consisting mainly of mineral and collagen. In normal humans, cortical bone constitutes approximately 80% of the human skeletal mass and trabecular bone approximately 20%. Bone surfaces may be undergoing formation or resorption, or they may be inactive. These processes occur throughout life in both cortical and trabecular bone. Bone remodeling is a surface phenomenon and in humans occurs on periosteal, endosteal, Haversian canal, and trabecular surfaces (1, 2). 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 two 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 5-10 times higher than cortical bone remodeling rates in the adult (1, 2). As is evident, tissue age is variable within the same human.

Osteoporosis & bone strength

Osteoporosis is an increasing public health problem. Currently osteoporosis is estimated to affect 200,000 people per year worldwide, costing the health care system over $ 10 billion per year in the US alone (3). Historically osteoporosis has been defined as a disease in which there is “too little bone, but what there is, is normal” (4). Recently, osteoporosis was defined as “a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk” (5). Despite the major efforts (6) being put into producing new therapies to prevent and reduce the bone loss that leads to osteoporosis, and to limit further loss when osteoporosis is recognized, the factors contributing to the fragility of bone are still being determined.

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 (7, 8), and there is considerable overlap in BMD between populations that do and do not develop fractures (9-11). It has been demonstrated that for a given bone mass an individual’s risk to fracture increases with age (12). Consistent with these findings, numerous investigators have shown that mechanical variables directly related to fracture risk are either independent or not totally accounted for by bone mass itself (13-18). 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 (9, 19-21). 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, remodeling activity, along with genetics, body size, environmental factors, and changes in bone tissue material (mineral maturity / crystallinity, collagen cross-links) properties (10, 11, 22).

Bone mineral

Bone mineral is a poorly crystalline hydroxyapatite [$Ca_5(PO_4)_3OH$] phase. Ion substitutions are abundant. For example, Na^{+1}, and Mg^{+2} are substituting Ca^{+2} ions, HPO_4^{-2} ions substituting the phosphate ions, Cl^{-1} and F^{-1} substituting OH^{-1}, and CO_3^{-2} substituting for either phosphate or hydroxyl groups. Once mineral is deposited in bone by osteoblasts, it is not a static moiety, but rather a dynamic one. Since it is bathed in aqueous biological fluids, the type and extent of these substitutions changes with time resulting in alterations of the mineral maturity, which is accompanied by changes in mineral crystallite size and /or shape (23, 24).

The contribution of mineral maturity, and crystallite size and shape to bone strength is very apparent in the case of fluoride treated bone (25-29).

In both animal models and in humans it has been reported that osteoporotic bone mineral characteristically consists of crystals which are larger and more perfect than in normal bone (30, 31), smaller and less perfect (32), or that there are no differences (33). Typically in these studies, tissues were homogenized prior to analysis, concealing the effect of spatial variations in mineral properties. Recently, utilizing techniques such as Small Angle X-ray Scattering (SAXS), and quantitative backscattered electron imaging (qBEI), the analysis of bone mineral (poorly crystalline hydroxyapatite) at the microscopic level and the contribution of mineral crystallinity (crystallite size) and maturity (chemical composition) to bone strength is being actively pursued (22, 26-29, 34-49). Based on such studies, models for the importance of mineral crystallite shape and size in determining bone strength have been put forth (50).

Bone collagen & collagen cross-links

The organic matrix of bone consists of collagen and a series of non-collagenous proteins and lipids. Some 85%-90% of the total bone protein consists of collagen fibers (51). Type I collagen, the principal component of the organic matrix of bone, as well as other connective tissues, is a large fibrous protein with a highly repetitive amino acid sequence $[Gly\ (glycine) - X - Y]_n$ (often X is proline and Y is hydroxyproline) (52-54). This repetitive sequence allows three polypeptide chains (called α chains; type I collagen is composed of two $\alpha 1$ and one $\alpha 2$ chains) to fold into a unique triple-helical structure. It consists of three domains: the $-NH_2$ terminal nontriple helical, the triple helical, and the –COOH terminal nontriple helical domains. The single uninterrupted triple helical domain represents more than 95% of the molecule.

The most distinct feature of type I collagen in mineralized tissues can be seen in its cross-linking chemistry and molecular packing structure (54). The intermolecular cross-linking provides the fibrillar matrices with various mechanical properties such as tensile strength and viscoelasticity. All the known cross-links of type I collagen are condensation products between the prosthetic groups of juxtaposed specific peptidyl residues of lysine (Lys), hydroxylysine (Hyl), and histidine (His). At present, seven major collagen cross-links have been established as naturally occurring intermolecular cross-links. They are (1) dehydrodihydroxylysinonorleucine (deH-DHLNL), (2) dehydrohydroxylysinonorleucine (deH-HLNL), (3) dehydrohistidinohydroxymerodesmosine (deH-HHMD), (4) pyridinoline (Pyr), (5) deoxypyridinoline (d-Pyr; lysyl analog of Pyr), (6) pyrrole, and (7) histidinohydroxylysinonorleucine (HHL). The first three are $NaBH_4$-reducible (their reduced forms are referred to as DHLNL, HLNL, and HHMD, respectively) and the rest are non-reducible compounds (54-57).

Altered collagen structure and inferior bone mechanical properties are encountered in the case of osteogenesis imperfecta, in both humans and animal models (35,58-68). The importance of collagen intermolecular cross-links to the mechanical performance of bone is also very apparent in the pyridoxine deficient chick animal model (69-71), as well as in lathyrism (72, 73).

Infrared spectroscopy

Molecular bonds are not stationary, but rather undergo motion such as twisting, bending, stretching, 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 atoms of a molecule. FTIR spectra provide information on all tissue components. The protein and mineral constituents produce intense, structure sensitive IR modes.

IR spectroscopy has been extensively utilized in the analysis of bone mineral (74-99). 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, stoichiometry) (78, 79, 84, 88-95, 97, 99).

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 frequency and intensity changes as a result of changes in protein secondary structure. The Amide I band is especially sensitive to secondary structures (100). 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 (100-106).

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, thus it was not possible to correlate the findings with the metabolic activity of bone surfaces (tissue age).

Infrared microspectroscopy

The coupling of an optical microscope with an infrared spectrometer in the early 1990's 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 bone surface metabolic activity (tissue age). The pioneering work of Drs Mendelsohn and Boskey (107-109) was later followed and expanded by them and others (38,110-124), 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 (116).

As informative as it may be, FTIR microspectroscopic analysis on instruments equipped with a single infrared detector was a time-consuming proposition as analysis of a single section required 2-3 days. The fairly recently available combination of an infrared focal-plane array (FPA) detector and a FTIR microscope is a powerful one for obtaining spectroscopic images with unprecedented image fidelity (125-128). The advantage of this technique lies in the fact that the spectra acquisition and processing time is shortened at least 1000-fold compared with conventional IR microspectroscopy. Use of a step-scanning FTIR spectrometer with an MCT array detector placed at an image focal plane of an IR microscope enables areas 400x400 μm^2 to be collected in less than 3-4 minutes at 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 (116, 117, 129-144).

Infrared microspectroscopy, bone strength & osteoporosis

Infrared microspectroscopic analysis of bone tissue from animal models and humans at equivalent anatomical locations gave great insight to the role of bone quality in determining bone strength (110, 112, 113, 115, 122, 124, 126, 132-134, 145). It became feasible to conclusively show differences in bone mineral maturity between normal and osteoporotic bone at equivalent anatomical locations (110, 112, 113, 122, 124).

Even more revealing was the analysis of the spatial variation in pyr and deH-DHLNL collagen cross-links in the same bones. It was shown that the ratio between these two major collagen cross-links was very different when osteoporotic and normal bones were compared in the area of trabecular bone with actively bone forming surfaces (141, 143). These data are in excellent agreement with recently published clinical observations that homocysteine blood serum level were elevated in patients with increased fracture risk (146-148). It is interesting to note that these differences were also observed between normal, and bone biopsies obtained from pre-menopausal women sustaining spontaneous fractures while having normal BMD and biochemical markers (143), suggesting that this might be a common factor / cause of fragile bone.

The effect of therapeutic protocols on bone quality has also been investigated (114, 115, 118, 121, 139, 140, 144). During these studies, it was discovered that when fracture risk and BMD were divergent, both mineral maturity and pyr / deH-DHLNL collagen cross-link ratio was correlating with fracture risk rather than BMD (137,144), further emphasizing the contribution of bone quality to its mechanical performance.

Future directions

Since the introduction of the Infrared Microspectroscopic analysis in the early 1990's, the debate rages whether it is a diagnostic tool. Although it provides a plethora of useful outcomes, it is our opinion that it is not 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 cases of fracturing patients whose "classical" risk indicators such as BMD and biochemical markers are normal (144).

On the other hand, it is a powerful research tool, affording unique insights into the pathophysiology of musculoskeletal diseases such as osteoporosis, osteogenesis imperfecta, Paget's disease, osteomalacia, ostepetrosis, osteosclerosis, etc. Its outcomes complement ones obtained through analyses such as histology, histomorphometry, biochemical markers, blood analysis, and BMD measurements, to provide detailed information on the mechanisms that result in healthy and diseased bone.

It is also a useful technique in deducing the changes in the spatial distribution variation of the mineral crystallite maturity and pyr and deH-DHLNL collagen cross-link ratio induced by various therapeutic protocols (114, 115, 118, 121, 139, 140, 142, 144), therefore it may be used in the future not only for evaluating the various therapeutic protocols but also assist in the design of more targeted ones.

Despite that both bone mineral crystallite maturity and pyr / deH-DHLNL collagen cross-link ratio have been shown to correlate well with bone strength, no calibration curve exists as all the cases reported thus far in the literature involved normal (100%) and diseased / fragile bone (0%). Since both mineral maturity and collagen cross-links do change long after they have been synthesized and deposited by the osteoblast as a consequence of tissue aging, establishing the threshold in the change in these two outcomes that results in mechanically inferior bone will be important as it will provide the calibration curve upon which bone strength may be predicted (when combined with the outcomes of other analyses), and help us discern between aging and disease.

One of the major advantages of Infrared Microspectroscopy 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 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 mm level) (28, 41-45), small angle x-ray scattering (providing precise information on the mineral crystallite size, shape and alignment to the collagen fibers) (22, 25, 27, 29, 34-37, 149, 150), and nanoidentation (providing information on the bone mechanical properties at discrete anatomical location with a spatial resolution of 1 μm) (151, 152), 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.

In conclusion, Infrared Microspectroscopy has proven to be a powerful tool in the establishment of parameters contributing to bone quality and thus bone strength. Nevertheless, more spectroscopic parameters describing the organic matrix should be derived in the future, and quantitation of these against bone strength should be achieved.

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