Infrared spectroscopic assessment of the inflammation-mediated osteoporosis (IMO) model applied to rabbit bone

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Abstract A model of osteoporosis based on induced inflammation (IMO) was applied to rabbit bones. The structural heterogeneity and molecular complexity of bone significantly affect bone mechanical properties. A tool like Fourier transform infrared spectroscopy, able to analyze both the inorganic and organic phase simultaneously, could provide compositional information regarding cortical and trabecular sections under normal and osteoporotic conditions. In this study, we assessed the mineral/matrix ratio, carbonate and phosphate content and labile (i.e., non-apatitic) species contribution to bone mineral and collagen cross-linking patterns. Clear differences were observed between cortical and trabecular bone regarding mineral and carbonate content. Induced inflammation lowers the mineral/matrix ratio and increases the overall carbonate accumulation. Elevated concentrations of labile species were detected in osteoporotic samples, especially in the trabecular sections. Collagen cross-linking patterns were indirectly observed through the $1660/1690\text{ cm}^{-1}$ ratio in the amide I band and a positive correlation was found with the mineralization index. Principal component analysis (PCA) applied to female samples successfully clustered trabecular and osteoporotic cases. The important role played by the phosphate ions was confirmed by corresponding loadings plots. The results suggest that the application of the IMO model to rabbit bones effectively alters bone remodeling and forms an osteoporotic bone matrix with a dissimilar composition compared to the normal one.

Keywords Fourier transform infrared spectroscopy · Bone composition · Osteoporosis · Inflammation-mediated osteoporosis · Apatite · Rabbit bone · PCA analysis

1 Introduction

Osteoporosis is a systemic skeletal disorder stemming from bone remodeling imbalance and leading to decreased bone strength and increased fracture risk [1]. Current clinical methods

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consider bone mineral density (BMD) as the criterion for antiosteoporotic treatment by measurements of bone strength conveyed as T-scores [2]. Although the T-score is currently the best validated surrogate marker of bone strength, it cannot sufficiently explain fracture incidence in elderly men and women [3]. The Rotterdam study [4] showed that the assessment of osteoporosis, based solely on densitometric measurements, i.e., T-score < -2.5, failed in almost half of the cases concerning non-vertebral fractures in postmenopausal women. Bone strength, as revealed by fracture resistance, is a complex property determined by both structural and compositional variables such as the spatial distribution of the bone mass and the inherent properties of the molecular groups that comprise the bone tissue [5, 6]. The term “bone quality” takes into account the biophysical and biochemical properties related to bone strength [7] in an effort to assess clinical outcomes that cannot be fully explained solely by BMD [8]. Although not precisely defined, bone quality correlates several parameters to fracture resistance called “intrinsic determinants” [9]. These include mineral composition, collagen quality, morphological factors, cellular activity, and microdamage [10–13]. Besides bone mass being a key determinant of bone strength [14], induced changes in the quality of bone mineral may significantly affect fracture susceptibility [15].

At the molecular level, bone mainly consists of an inorganic phase (calcium phosphate) deposited upon a soft organic matrix (98% w/v, mainly type I collagen). The mineral inorganic phase is a form of poorly crystalline carbonated hydroxyapatite ($\text{Ca}_{10}[\text{PO}_4]_6[\text{OH}]_2$) incorporating several impurities [16, 17] such as HPO_4^{2-} , CO_3^{2-} , Mg^{2+} , Na^+ , F^- and citrate, which are adsorbed onto the crystal surface and/or substituted in the lattice for Ca^{2+} , PO_4^{3-} and OH^- ions [18]. Impurities significantly increase solubility that is critical for phosphocalcic homeostasis and bone adaptation [19], while citrate stabilizes the apatite nanocrystals [20]. High values of mineral content [21] and grain size [22] are associated with increased bone stiffness and brittleness. The main component of the organic matrix, collagen type I, is a large fibrous protein consisting of three polypeptide chains composed of a $[\text{Gly-X-Y}]_n$ sequence where X and Y are proline and hydroxyproline, respectively [23]. These left-handed α -like helices fold into a unique right-handed triple-helical structure. Intermolecular cross-linking of type I collagen in mineralized tissues regulates tensile strength and viscoelasticity [24]. Non-collagenous proteins that are present in very low concentrations in the extracellular bone mainly affect bone remodeling [25], although recent studies suggest that they might also play a crucial role in the macroscopic mechanical properties of bone [26].

It is clear that this structural heterogeneity of bone considerably contributes to osseous properties such as stiffness, hardness, elasticity and overall strength. Thus, it is important to study the bone tissue as a whole, analyzing both the inorganic and organic phase. Fourier transform infrared (FTIR) spectroscopy is a valuable technique regarding this issue because it is non-destructive and highly sensitive to both the mineral and protein matrix of bone. In this study, we assess the inflammation-mediated osteoporosis (IMO) model applied to rabbit bone, using FTIR spectroscopy.

2 Materials and methods

2.1 Bone samples

Ten New Zealand white rabbits, 8 months of age, were housed into two equal gender groups. A group of five female rabbits of the same age was additionally included in the

study representing osteoporotic IMO cases. This was performed by injecting magnesium silicate (talcum) subcutaneously into the back of the animal and at distant sites from the skeleton to stimulate acute phase response by granulomatous reactions and accumulation of inflammatory cells [27]. The outcome is a decrease in osteoblast numbers and bone formation while osteoclasts and osteoclastic resorption are generally maintained. This is also the case in humans with chronic inflammatory diseases [28, 29]. It is reported that osteoporosis induced by the IMO method strongly affects the mineral phase by lowering the skeletal calcium/phosphorus ratio considerably [30] and it is developed independently of parathormone (PTH) and vitamin D metabolites. Energy dispersive X-ray spectroscopy experiments [31] found no evidence that Mg ions from talcum are transferred into the bone matrix displacing Ca ions. Therefore, IMO primarily affects osteoblast function as a result of the acute phase response following local inflammation, and spectral differences discussed in this study are indeed related to the induced osteoporosis.

Although the IMO model was originally designed for rats, there is evidence that it works equally well with rabbits [31]. Furthermore, unlike other mammalian model organisms such as rodents, rabbits achieve skeletal maturity at approximately 6 months, exhibiting noteworthy intracortical remodeling [32]. Peak bone mass is evident at 32–36 weeks and regular bone resorption and formation is established at this age [33]. Experimental osteoporosis has the disadvantage that mammalian species, except humans, do not develop fractures during their normal life span and therefore there is no animal model that precisely replicates the symptoms of spontaneous osteoporosis in humans. However, preventive and curative therapies for human osteoporosis can be reliably evaluated in satisfactory, well-defined animal models [34] given a careful experimental design. New Zealand white rabbits were specifically selected because of their short developmental period, fast bone turnover, and Haversian bone remodeling system similar to humans. Furthermore, the long bones of ageing rabbits feature a progressive, negative endosteal balance and a positive periosteal accrual that resembles the postmenopausal phase in women [35].

Cortical sections from the diaphysis of three different bone sites, femur, rear, and front tibias, all from the right side, were dissected out and cleaned free of soft tissue. Using the same procedure, the trabecular sections of rib bones were separated while the edges were discarded. Freeze-drying was performed to remove moisture. All animals were housed and bred in natural conditions, fed ad libitum and euthanized 21 days after the injection with anesthesia caused by ether. Care was taken throughout the experiments to ensure minimal pain or discomfort. The study was conducted under the approval of the Ioannina University Institutional Animal Care and Use Committee.

2.2 Spectral data collection

FTIR spectra were recorded with a Perkin–Elmer Spectrum GX FTIR system in the 4000–370 cm^{-1} range with 4 cm^{-1} signal resolution. Samples were prepared as KBr pellets consisting of 10% (by weight) bone powder. All spectra were baseline corrected and normalized prior to data analysis. Approximate peak locations were obtained using Savitsky–Golay first and second derivatives. Curve fitting and area integrations were performed with a non-linear least-squares algorithm (Levenberg–Marquardt), as this is implemented in OriginPro (OriginLab Corporation, Northampton, MA). Band shapes were considered as Gaussian and were linearly baseline corrected. Convergence was achieved in all cases ($R^2 = 0.99$). The computed area of each sub-band is reported as the percentage of the integrated area of the

whole band. Spectra manipulation was done with ACD/Specmanager (ACD Labs, Toronto, Canada).

2.3 Statistical analysis

Statistical analysis of the experimental parameters was carried out with PRISM software (GRAPHPAD software, San Diego, CA, USA). Data are reported as mean and standard deviation (SD). The statistical significance of differences of mean values was calculated by Kruskal-Wallis and Mann-Whitney tests. The statistical significance was determined at $p \leq 0.05$.

2.4 Multivariate analysis of spectra

Data processing of the IR spectra was performed in the $3000\text{--}400\text{ cm}^{-1}$ range to avoid statistical variations of the wide $\nu(\text{O-H})$ band ($3185\text{--}3575\text{ cm}^{-1}$), which is unrelated to the regions of interest of this study (Fig. 1). Data reduction of the 2601 variables (wavenumbers) was obtained using principal component analysis (PCA) algorithms. PCA is a statistical model that effectively removes the redundancy of the original data set by constructing linear combinations between variables into a few orthogonal uncorrelated principal components (PCs). Each component is constructed using weighted linear combinations of the original variables. These new composite variables describe most of the information contained in the original data in decreasing order. The first PC covers as much of the variance as possible; the second one is perpendicular to the first and explains the maximum possible of the remaining residual variance and so on. Scores are the coordinates related to PCs and loadings the

Fig. 1 Typical infrared spectrum of bone powder. Interesting absorption bands are indicated

related coefficients between the original variables and the new PCs. The spatial relationship of the data is visualized by plotting a two-dimensional score plot of the PCs in which clustering patterns are depicted. All spectra were normalized to the most intense band to minimize differences due to different amounts of bone sample or path length deviations. Discriminant analysis and classification schemes were carried out using The Unscrambler (CAMO Software AS, Norway) software package.

3 Results and discussion

3.1 FTIR spectra

3.1.1 Regions of interest

A typical infrared spectrum of a homogenized bone sample (trabecular rib sections) is depicted in Fig. 1. In all spectra, regions of interest concern common diagnostic vibrational bands revealing compositional information regarding organic and inorganic components of bone tissue. The intense band at $1720\text{--}1600\text{ cm}^{-1}$ is attributed to the absorption of the amide I functional group (peptide bond C=O stretching vibration and a minor contribution from the C-N stretching vibration of collagen). Below 1500 cm^{-1} is the fingerprint region of IR spectra where bands are due to types of motion allowed by the symmetry of the molecules in the mineral part of bone. The broad absorption band at $1200\text{--}900\text{ cm}^{-1}$ is assigned to the ν_1 (A_1) (symmetric stretching) and ν_3 (T_2) (antisymmetric stretching) normal modes of the apatitic phosphate ion [36]. The appearance of the ν_1 mode indicates that the geometry of the ion shifts to lower molecular symmetry since in such tetrahedral molecules only ν_3 and ν_4 are infrared active. This is also confirmed by the weak bands at 957 cm^{-1} and 473 cm^{-1} for the ν_1 , ν_2 dipoles, respectively. Thus, the appearance of the latent ν_1 dipole results in the removal of the degeneracy of the ν_3 mode. The doublet at 561 and 603 cm^{-1} is assigned to the ν_4 (T_2) (antisymmetric bending) mode. Deconvolution of this wide doublet to five sub-bands is readily related to non-apatitic and acid phosphate content. Hence, two bands near 621 cm^{-1} and a broad one near 532 cm^{-1} are attributed to non-apatitic phosphate and acid phosphate respectively while the fundamental ν_4 mode along with the band at 580 cm^{-1} corresponds to a crystalline apatitic environment (Fig. 2).

Regarding the structural parameters, IR data confirm that in biological apatite the phosphate ion adopts the C_{3v} point group symmetry instead of the T_d symmetry of the free ion, with three infrared active vibrations. Carbonate ions are also present in bone mineral (approximately 2–8% by weight) [37] and although bone contains significantly less carbonate than phosphate, carbonate largely affects the resorption of bone. The ion may occupy three different sites in biological hydroxyapatite: in monovalent anionic sites substituting for the hydroxyl group (A-type), in trivalent anionic sites substituting for the phosphate group (B-type), or on the surface of bone apatite crystals at random locations. The detailed configuration of the carbonate ions (A or B) in the lattice remains practically unknown due to the nano-dimensions of the apatite crystallites. The free carbonate ion possesses D_{3h} molecular symmetry and therefore exhibits four normal vibration modes ν_1 (A'_1) (symmetric stretching), ν_2 (A''_2) (out of plane bending) and two doubly degenerate modes, ν_3 , ν_4 (E'), of which only the three (ν_2 , ν_3 and ν_4) are infrared active. The ν_1 band is symmetry allowed under geometric distortion. The CO_3^{2-} ν_2 domain appears at 871 cm^{-1} and is deconvoluted to three sub-bands at 879 cm^{-1} , 871 cm^{-1} and 866 cm^{-1} attributed to the A-type, B-type, and labile (non-apatitic) carbonate content, respectively (Fig. 2). In the

Fig. 2 Curve fitting analysis of PO_4^{3-} ν_4 and CO_3^{2-} ν_2 absorption bands

same region, although minor, there is also absorption due to the HPO_4^{2-} group, which does not interfere with the quantitative estimations involving the carbonate ion [38]. Other bands of possible interest include the amide II band in the region $1600\text{--}1500\text{ cm}^{-1}$ (combination of the C-N stretch and N-H in-plane bending modes), amide III absorption at $1300\text{--}1220\text{ cm}^{-1}$ (C-N stretching and N-H in-plane bending vibrations), a weak band at 1 m^{-1} attributed to HPO_4^{2-} and a weak band at 664 cm^{-1} assigned to CO_3^{2-} ν_4 .

3.1.2 Band analysis and quantification

The overall mineral content (i.e., mineral/matrix ratio) can be estimated from the ratio of the integrated area of the ν_1 , ν_3 phosphate absorbance band to the integrated area of the protein

amide I absorbance band [39]. Mineral aggregation can also be assessed through the ratio of the $\text{PO}_4^{3-} \nu_4$ area ($650\text{--}500\text{ cm}^{-1}$) to the area of the amide I band [40]. We have concluded similar results from both methodologies, but the ones presented here are based on the second method, since curve fitting analysis of the ν_4 contour is more robust [41], has a lower absorption coefficient and is not susceptible to saturation effects. The mineral/matrix ratio is related to ash weight and is an approximate measure of BMD. However, its values are not comparable with BMD since it is a measure of mineral per amount of collagen present [42]. Yet, it reflects hyper- or hypo-mineralization that can deteriorate bone, primarily by altering the bonding between the bone mineral and the collagen matrix [43]. In general, models of osteoporosis [44] and osteoporotic tissues in humans [45, 46] are characterized by a decreased mineral/matrix ratio. No significant disparities ($p > 0.05$) were observed between the two sex groups regarding the degree of mineralization, confirming previous studies [47] that are also related to the Ca/P ratio as a critical mineralization biomarker [48, 49]. Trabecular bone apatite, represented by rib samples, shows decreased mineral deposited in the bone matrix in a highly significant manner ($p = 0.0002$) compared to the compact sections. IMO (osteoporotic) samples feature significantly ($0.03 < p < 0.04$) lower values than the control female samples although not significantly ($p > 0.05$) for the trabecular specimens. Trabecular sections constitute most of the bone tissue of the axial skeleton and the increased incidence of spinal and rib fractures under osteoporotic conditions is related to bone mass loss since the mineral is not heavily affected. On the contrary, results show that the mineral to matrix ratios of cortical bones are significantly reduced in all osteoporotic cases compared to the controls. Generally, cortical bones have a slow turnover rate, low porosity (5–10%), and their mineral is susceptible to less ionic substitution than the mineral within trabeculae. Thus, the IMO protocol followed in this study might be related to trabecularization of the endocortical surface leading to increased cortical porosity [50] (such analysis, though, is not possible with FTIR spectroscopy).

By second-derivative and curve-fitting analysis of the well-defined $\text{PO}_4^{3-} \nu_4$ band, we extracted information regarding the non-apatitic phosphate components. Similar to other studies [51], the bands centered at 532 cm^{-1} and 621 cm^{-1} (Fig. 2) remain practically constant among different sex and bone sites indicating approximately 15% of labile HPO_4^{2-} and 10% of labile PO_4^{3-} , respectively, pointing out the importance of such reactive species in the ion pool of the bone mineral. There is a significant increase in the concentrations of these ions regarding the osteoporotic samples that is more statistically important for the acid phosphate (Fig. 3) ($0.02 < p < 0.03$ and $p = 0.008$ regarding the HPO_4^{2-} increase in cortical and trabecular bones, respectively). This is probably attributable to the replacement of PO_4^{3-} by HPO_4^{2-} ions in the osteoporotic bones and the creation of an anionic vacancy in the lattice, which is either compensated for by the removal of a Ca^{2+} cation or by CO_3^{2-} substitution due to the increase of the unstable carbonate ions mostly in trabecular regions.

The carbonate/mineral ratio was assessed as the integrated area of the carbonate peak divided by the phosphate peak. This parameter is inverse linearly related to the elastic modulus of bone. Analogously, total carbonate accumulation is calculated by the carbonate to amide I peak ratio. Carbonate-specific parameters are correlated to turnover rate, remodeling activity [52], and mineral dissolution [53]. The carbonate/mineral ratio variation remains statistically minimal throughout all samples (not shown). On the other hand, the carbonate/amide I ratio is significantly lower ($0.002 < p < 0.004$) in trabecular than cortical samples for all populations. This difference is consistent with the slow turnover rate of the cortical bone [54]. The carbonate content increases in osteoporotic samples compared to the controls, albeit not statistically significantly ($0.1 < p < 0.4$). This parameter, however, is rather unstable since in various studies it either increases [55, 56]

or decreases [52, 57], possibly because it refers to the total content and neglects the specific contribution of each type. The type of carbonate substitution was calculated by deconvolution of the CO_3^{2-} ν_2 contour (Fig. 2). The results confirm the fact that biological apatites are mainly B-type carbonate apatites with small fractions of A-type impurities (approximately 15%) and this ratio remains practically constant after the completion of healthy bone mineralization [58]. On the other hand, there is a significant ($0.02 < p < 0.03$) decrease of non-apatitic carbonate locations of trabecular sections, signifying these as more immature compared to the cortical ones. Labile carbonate values for IMO samples show a small deviation among all bone sites (Fig. 3) and a significant increase for rib sections. Non-apatitic environments in bone mineral play a crucial role due to their high reactivity and thus their enhanced biological functionality [38]. In this case, the higher values noted for the labile species may be related to the inflammation-affected increased bone turnover rates [59].

Collagen maturity is estimated by the $1660/1690\text{ cm}^{-1}$ sub-bands ratio of the amide I band. This ratio has been related with the degree of pyridinium (mature) relative to reducible (immature) types of collagen cross-links [60]. Reduced concentrations of collagen cross-links are associated with reduced tensile strength [61]. In rats, the cortical bone has a higher ratio value than trabecular ones [62], but this is not repeated in our case where rabbit bones exhibit non-significant variations. However, in osteoporotic trabecular samples, a significant increase ($p = 0.004$) of the $1660/1690\text{ cm}^{-1}$ ratio was observed, indicating an increase in the non-reducible cross-links consistent with previous studies [63, 64]. Conversely, in the cortical bone regions, there is an increasing trend that is significant ($p = 0.02$) only for the front tibia (not shown). The results suggest that there is a particular contribution of collagen cross-linking to the properties of each bone site. Knott and Bailey [65] have also shown that avian cortical and trabecular sections from the humerus and tibia have different cross-linking patterns. Detailed spectra processing shows that the amide I band is a composite of collagen molecular groups, water molecules, and non-collagenous proteins. Given the non-negligible interference of the non-collagenous molecules as well as the different extinction coefficients of water and collagen, there is a great uncertainty regarding the ratio measured. In addition, recent studies have correlated the $1660/1690\text{ cm}^{-1}$ ratio to mineral maturity and to the degree of mineralization rather than to the modification of the enzymatic cross-links [66]. Thus, such a measurement could reflect the change in the secondary structure of collagen related to the mineralization process as well as to the dehydration of the mineral phase. Pearson correlation calculations showed a significant positive correlation ($p = 0.03$) with 10% variance shared between the mineralization degree and the $1660/1690\text{ cm}^{-1}$ ratio ($r^2 = 0.105$) despite the inverse relation of the variables found for the trabecular samples.

3.1.3 Multivariate analysis

Discriminant analysis is based on every distinct wavenumber of each spectrum to detect even a minor modification between samples regarding their IR active molecular groups. No discrimination was possible for different sex populations. Furthermore, clustering patterns are not feasible for bone sites in accordance with previous results [67], supporting the idea that the molecular groups probed by IR spectroscopy have the same effect on the composition of bones. However, as is evident from Fig. 3, trabecular rib sections are deficient in degree of mineralization and carbonate content. Regarding female populations (normal and IMO) only, rib samples are clearly separated by the negative correlation of the PC1 score, which explains 78% of the total variance. They also feature positive PC1

Fig. 3 Parameters studied across bone sites and between normal and osteoporotic samples by FTIR spectroscopy (*M* male, *F* female, *FTIB* front tibia, *FEM* femur, *RTIB* rear tibia, *RIB* rib). SDs plotted as error bars. Asterisks denote statistical difference (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

Fig. 4 Clustering schemes with PCA scores and corresponding loadings plots (female samples)

loadings near the phosphate ν_4 band and at a very wide contour covering both carbonate ν_2 and phosphate ν_1 , ν_3 bands (Fig. 4a). Hotelling's T2 ellipse, revealing three potential outliers lying outside the 95% confidence, is also shown. An additional 10% of the variance is explained by PC2, which features significant positive contributions in the loadings plot at the phosphate and amide I regions.

Analysis demonstrated that higher-order PCs (2, 4) are able to explain 12% of the total variance, effectively discriminating IMO and normal female samples (Fig. 4b). The regions that contribute most to the clustering observed in the scores plot are the phosphate and amide bands, corroborating the conclusions drawn from examination of Fig. 3 and previous analysis where the largest variation for osteoporotic samples is observed for labile phosphate and collagen.

4 Conclusions

Vibration frequencies manifested as absorption intensities, band shapes, and positions, are very sensitive to the molecular environment of the atoms involved in the vibration. Infrared spectral details of bone tissue provide a lot of information regarding its compositional characteristics in the inorganic and the organic phase. In this study, we examined how the IMO model applies to rabbit cortical and trabecular bones and we successfully differentiated normal and osteoporotic samples based on FTIR spectroscopy. Induced inflammation increases the overall carbonate accumulation in both cortical and trabecular sections, probably due to the increased turnover causing higher concentrations of immature bone. Results from the phosphate/amide I and carbonate/amide I ratios demonstrated that the cortical bone sites exhibit higher values when compared to the trabecular bone sites. The labile acid phosphate content increases considerably in IMO samples also, suggesting accelerating bone turnover. Different cross-linking patterns for IMO samples are indirectly observed through the $1660/1690\text{ cm}^{-1}$ ratio for cortical and trabecular sections and a positive correlation was confirmed with the mineralization index. Results may suggest that, apart from the disturbed bone remodeling, the osteoporotic bone matrix formed is also

different from the normal one, although it is not clear if such changes are triggered from increased bone turnover or from more complex pathophysiological effects. Multivariate statistical analysis successfully clustered female samples according to bone type (cortical vs. trabecular) and induced osteoporotic samples.

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