Spectroscopic Assessment of Normal Cortical Bone: Differences in Relation to Bone Site and Sex

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Bone is a highly complex, composite tissue and its properties normally vary with age, type, and disorders. Fourier transform infrared spectroscopy and energy-dispersive X-ray spectroscopy techniques were used to study the effect of bone sites and sex to mineral and matrix content and composition. The results show that in rats, all inorganic phases consist of poorly crystalline B-type carbonated apatite, while overall mineralization and carbonate content is virtually unaffected among samples. Statistically significant differences were detected for the nonapatitic environments of acid phosphate and carbonate content. The mean values for the Ca/P ratio point to an increasing trend from tibia to forearm, and to femoral sections.

KEYWORDS: Fourier transform infrared spectroscopy (FTIR), vibrational spectroscopy, energy-dispersive X-ray spectroscopy (EDX), SEM, bone mineral, cortical bone, Ca/P ratio

INTRODUCTION

Bone, at the molecular level, is described as a composite material consisting of calcium phosphate crystals, water, and soft organic material, mostly collagen, which surrounds the mineral crystals by an exceptionally dense filling[1]. These constituents are commonly referred to as mineral and matrix components, and are distributed in diverse patterns among different bone types. The mineral phase in calcified tissues plays a significant role, mainly because it strongly affects their strength and quality. Qualitative parameters, such as mineral crystal perfection, composition, and size, vary considerably in relation to bone age, type, site, and also to bone metabolic diseases. Mechanical strength mainly depends on the state of cortical bone[2]. Stress tests on excised femoral necks have shown that the cortex contributes 40–60% of the overall strength[3]. In addition, studies on stress distributions associated with normal gait showed that the majority of load in the femoral neck region is carried by cortical bone[4]. Thus, in the present work, the effect of bone site and sex on mineral content and composition of rat cortical bone was studied.

For the nondestructive examination of biological tissues, among other techniques, Fourier transform infrared (FTIR) spectroscopy is often used. This technique is particularly useful because it relies on objective criteria such as changes of the IR absorption frequency and intensity in various functional groups of biological molecules. For bone, FTIR spectroscopy can be used to scrutinize mineralization,

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maturity of mineral crystal and collagen, carbonate accumulation and environment, as well as acid phosphate content[5]. Phosphate, carbonate, and hydrogenophosphate ions have an important effect on the formation and maturation of bone mineral since their relative concentration indicates the stoichiometric evolution of poorly crystalline biological apatite. This evolution proceeds by the transformation of the initial, rich-in-nonapatitic-species phase into a stable, more crystalline, carbonate apatite.

The relative content of calcium against phosphorus is generally accepted as a regulator to maintain mineral homeostasis and bone metabolism[6]. It has been proposed that changes of the Ca/P ratio are more crucial for the valuation of bone health than variations of the concentrations of either Ca or P[7,8,9], as these deviations are not necessarily in mutual association. Thus, a method able to access the Ca/P ratio could lead to a greater understanding of the role played by these elements. Energy-dispersive X-ray (EDX) spectroscopy is a sensitive qualitative and semi-quantitative procedure for the assessment of the mineral content variations in microscopic regions of bone[10]. Furthermore, the range of Ca/P values in response to the parameters studied can be correlated with the spectroscopic results.

MATERIALS AND METHODS

Animals – Bone Samples

Ten female and male Wistar rats, 8 months of age, were used for the present study. Animals were housed and bred in natural conditions and euthanized under light ether anesthesia. Cortical sections from three different bone sites, femur, tibia, and forearm (ulna), all from the right side, were dissected out and cleaned free of soft tissue. There were no signs of metabolic alterations of the bone tissue. To facilitate removal of loosely bound water, the samples were freeze dried. All studies are approved by the Ioannina University Institutional Animal Care and Use Committee.

FTIR Spectral Data Collection

FTIR spectra were recorded on a Perkin-Elmer Spectrum GX FTIR system with samples prepared as KBr pellets, in the 4000- to 370-cm⁻¹ range, at a resolution of 4 cm⁻¹. The resolution of the spectra was retained, as no smoothing algorithms were applied prior to deconvolution analysis. Pellets were prepared by mixing KBr and bone powder (10% by weight) in a mortar and pestle, and pressed in a 13-mm pellet die. FTIR spectra were acquired immediately after lyophilization.

EDX Data Collection

The samples were examined by scanning electron microscopy (SEM) in a Jeol JSM 5600 system configured with an EDX detector operating at 20-kV accelerating voltage and 20-mm working distance. The EDX data were compiled for analysis using the Link ISIS system (Oxford Instruments, U.K.). The Ca/P ratio was calculated using the accompanying ZAF-correction software (SEMQuant, Oxford Instruments). A standard sample with similar Ca/P ratio was analyzed prior to each EDX session. Due to the fact that the scanning electron microscope operates at vacuum conditions, the tissue was partially dehydrated with an organic, water-miscible solvent. This was achieved by upgrading a 70% solution of EtOH to absolute in ascending concentrations. Then, the tissues were slightly polished, further dehydrated by freeze drying, and coated with a conductive carbon layer using a JEOL JEE-4X Vacuum Evaporator. The electron beam can induce significant damage on biological substances[11], so we performed excessive tests on operating conditions. Thus, the magnification was kept low (×50 – ×110) and each spectrum was collected twice every 60 sec until a total collection time of 120 sec was reached. No

significant discrepancies were found between each measurement concerning the Ca/P ratio, which was finally estimated from the mean value of two different locations of the specimen.

Data Analysis

Statistical analysis for the parameters under study was carried out utilizing Graph Pad INSTAT software (GraphPad Software, San Diego, CA). Data are reported as mean $\pm$ SD. Evaluation of statistical significance of differences of mean values was performed using nonparametric, one-way ANOVA (Kruskal-Wallis analysis of ranks) for the three bone site groups and Mann-Whitney t -test for the two populations. Even though the physical substrate of the experiment designates Gaussian distribution of the data, we followed rigid statistical methods owing to the small sample size. Statistical significance was considered at $p < 0.05$. Curve fitting and area integrations were performed using OriginPro (OriginLab Corporation, Northampton, MA) and the minimization was based on the Levenberg-Marquardt algorithm. Band shapes were always considered as Gaussian and were baseline corrected. The convergence criterion was met in all cases with $R^2 = 0.99$. 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) and peak positions were originated by second derivative analysis using the Savitsky-Golay method (second-order polynomial with 19 data points).

RESULTS AND DISCUSSION

FTIR Spectra

A typical processed IR spectrum of the homogenized bone sample is depicted in Fig. 1, where regions of interest are also shown.

FIGURE 1. Typical IR spectra of homogenized bone samples. Regions of interest are indicated.

The measured spectra do not differ appreciably ($\pm 1 \text{ cm}^{-1}$) and, consequently, some common diagnostic vibrational bands are reported in Table 1.

TABLE 1
Bands Assignments[12,13]

Assignment	IR Frequency (cm^{-1})
$\text{PO}_4^{3-} \nu_2$	473w
$\text{PO}_4^{3-} \nu_4$	561s, 603s ^a
$\text{CO}_3^{2-} \nu_4$	669vw
$\text{CO}_3^{2-} \nu_2$	871s ^a
$\text{PO}_4^{3-} \nu_1$	957sh
$\text{HPO}_4^{2-} \nu_3$	1163vw
$\text{PO}_4^{3-} \nu_3$	1027s, 1101w
Amide III	1236s
CH_2 wagging	1340s
νCOO^-	1398s
$\delta_{\text{as}}\text{CH}_3$, $\text{CO}_3^{2-} \nu_3$	1460s ^b
$\nu_{\text{as}}\text{COO}^-$	1510s ^b
Amide II $\delta(\text{N-H}) \nu(\text{C-N})$ (C-N stretching and N-H in-plane bending)	1546s–1562s
Amide I (C=O stretch)	1649s
C=O nonionized	1735w
νCH_2	2885sh
$\nu_{\text{as}}\text{CH}_2$	2938w
$\nu_{\text{as}}\text{CH}_3$	2963mw
$\nu(\text{C-H})$ Amide B	3075w
$\nu(\text{O-H})$, $\nu(\text{N-H})$ (Amide A)	3185s–3575s

^aIR envelope; ^bpoorly resolvable.

In the functional group region, the intense band at 1649 cm^{-1} is attributed to the absorption of the Amide I functional group (peptide C=O stretching vibration of the collagen), which represents the organic matrix of bone since the vast percentage of the protein content is collagen (mainly type I). It also shows that the main helix band is dominated by the secondary structure of collagen[14]. The Amide II band near 1550 cm^{-1} is due to a combination of the C-N stretch and N-H in-plane bending modes. In the fingerprint region, the broad and strong absorption band at $900\text{--}1200 \text{ cm}^{-1}$ is assigned to the ν_1 and ν_3 normal modes of the apatitic phosphate ion, the most profuse ion in bone mineral. The free ion exhibits four fundamental modes of vibrations: ν_1 (A_1) (symmetric stretching), ν_2 (E) (symmetric bending), ν_3 (T_2) (antisymmetric stretching), and ν_4 (T_2) (antisymmetric bending)[15,16]. Due to the fact that only the triply degenerate vibration species of the tetrahedral molecules are IR active, only ν_3 and ν_4 vibration modes should be observed. Nevertheless, the other two modes, ν_1 and ν_2 , become observable as the geometrical parameters shift to lower molecular symmetry. In our case, the symmetry of the phosphate ion has obviously been lowered to a point group compatible with ν_1 , ν_2 modes; this is evident from the weak bands at 957 and 473 cm^{-1} , respectively. The appearance of the latent ν_1 dipole, as well as the interaction with the neighboring atoms, results in the removal of the degeneracy of the ν_3 mode. Thus, the wide range of $960\text{--}1140 \text{ cm}^{-1}$ may possibly be deconvoluted by a maximum of three absorption bands. The doublet at 561 and 603 cm^{-1} is assigned to the ν_4 bending mode. It is of significance to mention that no hydroxide ion bands appear in this region, typically as a weak shoulder at 630 cm^{-1} , as is the case with (mostly synthetic)

hydroxyapatites. This absence of the OH⁻ band in the spectra of bone samples probably does not denote the complete deficiency of hydroxide ions in bone mineral. This is further supported by the data presented in Table 1, plus other studies[17,18]. The $\nu(\text{OH})$ band is shown in Fig. 1 as a wide contour and is most probably attributed to embedded water molecules and not to lattice hydroxyl ions. The lack or not of hydroxyl ions in bone apatite is still under investigation and a matter of dispute[19] among researchers. Given the number of bands as well as crystallographic data[20], we conclude that the phosphate ion of the biological apatite adopts the C_{3v} point group symmetry instead of the T_d symmetry of the free ion, giving three formal IR active vibrations. Further complications, such as HPO_4^{2-} ions and the presence of vacancies in the lattice, expand the number of possible convoluted sub-bands of the wide phosphate moiety. The presence of the band at 1163 cm^{-1} is attributed to the HPO_4^{2-} related to nonstoichiometric hydroxyapatite. The latter and its content decrease have been connected with the high proportion of organic content in bone[17]. The very low intensity of this absorption band is consistent with the age of the population sample, as it is reported that although nonapatitic acid phosphate environments diminish with age, some fraction of acid phosphate ion is present at different levels of bone development[21]. The carbonate ion is present in bone mineral approximately 2–8% by weight[22] and its concentration is also age dependent[23]. The free 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 ν_3 , ν_4 (E'), of which only the latter three are IR active. As is the case with the phosphate ion, the ν_1 band is allowed when the symmetry lowers on the lattice carbonate location, but all the same, it is hidden beneath the ν_3 phosphate band. The ion may occupy three different sites in bone mineral: 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 ion (A or B) on either site in the lattice remains unknown, mainly due to the minute crystallite size of the apatite. On the other hand, A-type carbonate apatite features IR absorption near 879 cm^{-1} , while the B-type is at 871 cm^{-1} . A third band at 866 cm^{-1} is assigned to labile (nonapatitic) carbonate, the concentration of which decreases as bone matures, although the overall carbonate content increases[24]. All these bands belong to the CO_3^{2-} ν_2 domain and they are observable by applying Fourier self-deconvolution to the spectra. The stronger IR band, CO_3^{2-} ν_3 , is severely overlapped by intense absorption bands of organic material (Table 1), which makes this spectra area ineffective for further consideration. The ν_4 fundamental shows a single very weak band at 669 cm^{-1} . As a result, only the ν_2 mode is suitable for quantitative calculations of carbonates in the bone mineral. Absorption of the HPO_4^{2-} group at the same region produces a broad band that can be neglected, since it has been shown that it does not interfere with the quantitative estimations involving the carbonate ion[25].

IR Analysis

Overall mineral concentration can be estimated by the ratio of the integrated area of the ν_1 , ν_3 phosphate absorbance bands to the integrated area of the protein Amide I absorbance band[26,27].

An alternative method to assess mineral aggregation is through the ratio of the PO_4^{3-} ν_4 area ($500\text{--}650\text{ cm}^{-1}$) to the integrated area ($1600\text{--}1700\text{ cm}^{-1}$) of the Amide I band[28]. Although analogous experimental results were obtained by either procedure, curve fitting analysis of the ν_4 contour is more robust, since this can be fit to fewer components[29]. For this reason, content related the phosphate group was obtained from the PO_4^{3-} ν_4 spectral region.

The mineralization results are tabulated in the first column of Table 2, where there is a comparison between sex and bone sites. No significant disparities were observed between the two groups ($p > 0.05$), although standard deviation calculation showed that distribution of values for the male population is more scattered than the female population, resulting in a high coefficient of variation (10, 16, and 14% for tibia, femur, and forearm sections, respectively). To confirm the outcome of this method, simple area integrations of the ν_1 , ν_3 curve was performed for all samples. No significant discrepancies were identified among the two groups or the three bone sites. By means of second derivative and curve fitting analysis of

TABLE 2
Summary of Parameters Analyzed through Vibrational Spectroscopic Analysis

	Mineral	Labile HPO_4^{2-}	Labile PO_4^{3-}	Carbonate	Type A/Type B	Labile CO_3^{2-} /Type B	Collagen Maturity
Tibia							
M*	2.75 ± 0.26	0.15 ± 0.02	0.09 ± 0.005	0.16 ± 0.02	0.16 ± 0.09	0.31 ± 0.03	2.79 ± 0.59
F*	2.66 ± 0.22	0.23 ± 0.02	0.08 ± 0.008	0.17 ± 0.02	0.15 ± 0.01	0.36 ± 0.05	2.37 ± 0.58
Femur							
M	2.88 ± 0.47	0.19 ± 0.03	0.08 ± 0.02	0.18 ± 0.03	0.15 ± 0.06	0.36 ± 0.06	2.81 ± 0.77
F	2.89 ± 0.12	0.28 ± 0.02	0.07 ± 0.002	0.16 ± 0.02	0.17 ± 0.02	0.39 ± 0.05	2.73 ± 0.90
Forearm							
M	2.93 ± 0.41	0.20 ± 0.08	0.08 ± 0.01	0.20 ± 0.05	0.14 ± 0.02	0.34 ± 0.04	2.30 ± 0.39
F	2.73 ± 0.12	0.23 ± 0.05	0.07 ± 0.002	0.17 ± 0.02	0.15 ± 0.01	0.38 ± 0.07	2.24 ± 0.37

* M, male; F, female.

the well-defined PO_4^{3-} ν_4 band, acid phosphate information was extracted from the sub-bands intensity ratio (Fig. 2). The wide contour was decomposed with the input of five Gaussian-shaped bands. In order to avoid inconsistencies between variable sets, we kept the baseline linear and the position of the band at 603 cm^{-1} locked. All other parameters (peak center, width, and area) were left free. Two bands near $621 (\pm 2) \text{ cm}^{-1}$ and a broad one at $532 (\pm 8) \text{ cm}^{-1}$ are attributed to nonapatitic phosphate and acid phosphate, while the fundamental PO_4^{3-} ν_4 mode appears at 561 and 603 cm^{-1} , corresponding to crystalline apatitic environment along with the band at 580 cm^{-1} .

In accordance with the results of other groups[30], the band area at 621 cm^{-1} remains practically constant, indicating 8–9% of labile PO_4^{3-} in all cases (different population and/or bone site), showing that such reactive species might play an important role in the ion pool of the bone mineral throughout all ages[31]. The wide band centered between 524 and 540 cm^{-1} is assigned to acid phosphate content where HPO_4^{2-} ions are readily substitutable by carbonate groups. There is an increasing trend ($p = 0.08$) of the labile HPO_4^{2-} from male to female population (Table 2). This finding is evaluated as particularly significant ($p < 0.01$) for tibia and femoral sites, and clearly demonstrates that female bone mineral features inherent intracrystalline disorder compared to that of males. This disorder in the female group may also be attributed to differences in the relative bone sizes of the two populations.

Carbonate concentration was measured by the CO_3^{2-} ν_2 ($855\text{--}890 \text{ cm}^{-1}$) to Amide I ratio. Nonsignificant variations ($p > 0.05$) were observed involving either different populations or bone sites. The application of curve fitting of these two areas, respectively, provides valuable information about the substitution effect for the three types of carbonate and collagen cross-linking alterations related with the tensile strength and viscoelasticity of bone[32,33,34]. Using curve fitting procedures, we evaluated the relative site of carbonate substitution for all the homogenized bone samples. Second derivative analysis revealed three sub-bands for the CO_3^{2-} ν_2 contour located at 879 , 871 , and 866 cm^{-1} . The first two bands are assigned to apatitic locations of the carbonate ion in the two anionic sites of the structure normally occupied by phosphate and hydroxyl ions, respectively.

The results (Table 2) agree with those of other workers[35] that biological apatites are mainly B-type carbonate apatites with small fractions of A-type impurities (ca. 15%) and this ratio remains practically constant after the completion of bone mineralization. Thus, the prevailing substitution occurs for the phosphate ion and this is connected with an increased stability of the apatite itself[22]. Upon fitting, there is a slight shift of the labile CO_3^{2-} sub-band to 862 cm^{-1} , indicating a sort of interaction and minor changes in the environment of the molecule. The ratio between labile and B-type carbonate was low and

FIGURE 2. Typical curve fitting analysis of $\text{PO}_4^{3-} \nu_4$, $\text{CO}_3^{2-} \nu_2$, and Amide I IR bands.

analogous to mature bone mineral. There is an increasing tendency of this ratio between males and females that is statistically significant ($p < 0.05$). The means are slightly dissimilar among sites of the same sex, while naturally the peak area of the nonapatitic carbonate ion is generally decreasing upon bone maturation. We therefore conclude that in females, these carbonate species exist to an increased concentration, suggesting an inadequately structured area of the apatite crystal lattice.

There is a positive correlation ($p = 0.01$) between the unstable carbonate location and the acid phosphate content found for all samples. Both ions exhibit a statistically significant increasing trend between populations and not among different bone sites. In Fig. 3, the linear regression plot of the two ions ($R = 0.83$; slope = 0.59 ± 0.13 ; Y-int: 0.23 ± 0.03) is presented involving the medians for the two populations.

FIGURE 3. Linear relationship between labile carbonate and acid phosphate content of homogenized bone samples. The curves represent the 95% confidence band of the best fit line (● Male, □ Female).

The $1660/1690\text{ cm}^{-1}$ ratio of the Amide I band has been related with the relative amount of nonreducible (mature) to reducible (immature) types of collagen cross-links[36]. This ratio has been shown to increase with collagen maturity and with the degree of mineralization. Fourier self-deconvolution as well as second derivative analysis was also utilized in this case to separate the two peaks. In accordance with our findings about mineral concentration, this ratio varies nonsignificantly ($p > 0.05$) among specimens.

EDX Measurements

Table 3 summarizes the results from the analysis of the bulk Ca/P atomic ratios of the cortical bone for different bone sites and sex.

TABLE 3
Bulk Values of the Ca/P Ratio Measured
by EDX Spectroscopy

Bone Type	Mean Ca/P	
	Male	Female
Tibia	1.85 ± 0.10	1.84 ± 0.06
Femur	1.96 ± 0.11	1.93 ± 0.09
Forearm	1.95 ± 0.12	1.91 ± 0.13

If cortical bone contained only mineral (hydroxyapatite), a stable ratio $\text{Ca/P} = 2.16$ should exist. Nevertheless, cortical bone contains 30–40% organic matter, which affects the Ca/P ratio from that of pure hydroxyapatite, resulting in Ca-deficient apatite[37]. Medians involving all three bone sites do not vary significantly ($0.06 < p < 0.4$), while there is no statistical disparity between the two populations ($0.5 < p < 0.8$). Statistical differences comparing tibia and femoral sections are significant ($p = 0.04$) in accordance with previous findings[8,9] where synchrotron microCT was used. Other researchers have also noted differences in trabecular bone composition among skeletal sites[38]. Factors that may contribute to such variations are different bone cell populations, differential biomechanical tension, and local differences in the blood supply of bone. As trabecular is more metabolically active than cortical bone, it demonstrates higher divergence in composition between bone sites than in our case. The lower Ca/P ratio for the female group, albeit not particularly significant, is explained by the higher content of HPO_4^{2-} , as discussed in the previous section.

CONCLUSIONS

It is very interesting to mention the discrimination observed by the different experimental procedures followed in this study. FTIR spectroscopy, which probes functional groups, classified the samples based on sex, while EDX spectroscopy, which probes elemental analysis, classified the samples based on bone sites. The discrepancies found among bone sites are probably due to variations of Ca concentration. Differences found between the two populations are likely due to instabilities of the carbonate and acid phosphate content originated from microscopic (molecular) or macroscopic (bone length) factors. In particular, the female population shows increased concentrations of the latter acidic group. The increase in monohydrogen phosphate and the replacement of some PO_4^{3-} by HPO_4^{2-} ions, even though it creates an anionic vacancy, are not primarily compensated by the removal of Ca^{2+} from the lattice[39], as is evident from EDX measurements; according to IR spectra, this is most probably due to CO_3^{2-} substitution following the increase of the unstable carbonate ions.

The deviation of the Ca/P ratio among bone sites follows the same trend with the variation of mineral content. The local nonapatitic environments of both carbonate and phosphate ions seem to play a crucial role in the ion pool of bone mineral. Upon maturation, these ions exist in noticeable concentration, enforcing the hypothesis of carrying out biological and mechanical functions by their enhanced reactivity[30]. The same argument stands for nonapatitic acid phosphate ions that, in general, decrease rapidly with carbonate accretion. Although many types of ionic substitutions in bone apatite lattice are critical to its crystallite size and dissolution rate, as far as the mature bone is concerned, the anionic substitution of B-type predominates. Maturity of collagen fibrils as extracted from FTIR data is in accordance with the age of the samples.

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