Investigation of composition and structure of spongy and hard bone tissue using FTIR spectroscopy, XRD and SEM

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Abstract Valuable structural and chemical features can be obtained for spongy and hard bone by infrared spectroscopy and X-ray diffraction. A better understanding of chemical and structural differences between spongy and hard bone is a very important contributor to bone quality. Our data according to IR data showed that the collagen cross-links occurred to be higher in spongy bone, and crystallinity was lower in spongy bone. Deconvolution of the infrared band near 870 cm⁻¹ reveals evidence for A2-type carbonate substitution on hydroxyapatite of spongy bone in addition to the A and B type carbonate substitution that are also found in hard bone. IR and XRD data confirmed the results of each other since full width at half maximum of 002-apatite pattern of XRD showed that the crystallinity was lower in spongy bone. The microstructure was examined by using scanning electron microscope and the result showed that the lattice of thin threads in spongy bone and is less dense than hard bone.

1. Introduction

A bone is a rigid organ that comprises part of the vertebrate skeleton. Bones performs many major functions, such as; support and protect the various organs of the body, production of red and white blood cells, storage of minerals, and endocrine regulation. Hard bone, is also known as cortical bone, is the dense outer surface of bone that forms a protective layer around the internal cavity, and it makes up nearly 80% of skeletal mass and is imperative to body structure and weight bearing because of its high resistance to bending and torsion. Spongy bone also is known as cancellous bone, is one of the two types of bone tissue found in the human body. Spongy bone is very porous and contains red bone marrow, where blood cells are made. It is weaker and easier to fracture than hard bone. Bone is a heterogeneous composite material of a mineral phase, hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂) and an organic phase, (∼90% collagen type I, ∼5% noncollagenous proteins, ∼2% lipids by weight) and water [1-3]. Chemical composition is an important contributor to bone quality, a term that encompasses the effects of architecture, composition and remodeling dynamics [4].The mineral component of human bone is carbonated hydroxyapatite because of the tendency of CO₃²⁻ to substitute for OH⁻ and PO₄³⁻, with the existence of hydroxyl groups being possible theoretically but difficult to detect [5]. Carbonate increases the solubility of the apatite crystal [6], with levels in the crystal lattice commonly between 2 and 7.4 wt %, the variation of which is largely dependent on age [7, 8].There are many techniques to determine the composition and quantity of mineral present in bone [9].Vibrational spectroscopy is a powerful technique which has been used widely to characterize the bone matrix providing information about the bone quality and its composition on the microstructural level[10-13].
Age-dependent changes in FTIR spectral parameters of healthy and diseased human bone have earlier been summarized by Boskey and Mendelsohn [14]. Based on the quantitative analysis, it has been shown that the amide I and amide II components of proteins undergo frequency and intensity change as a result of changes in the protein secondary structure [15]. Moreover, X-ray diffraction is a useful non-destructive technique widely used for the characterization of structural properties. De Jong [16] was the first to apply x-ray diffraction on bone and establish the inorganic part of bone as hydroxyapatite. The aim of this study was to compare chemical and structural properties of hard and spongy bone. Both IR and XRD features were analyzed to confirm the findings.

2. Materials and methods
Our samples consist of bone pellets. Hard tissue was obtained from fresh lamb bones of the chest. After peeling off the soft tissue, it was incubated overnight to give a strong and crust form. Using a mortar mould the bones were carefully broken into smaller fragments, the smaller fragments were carefully separated from the bone marrow to avoid interference and a coffee maker grinder was used to grind the bones into powder. 1g of PEG was dissolved into 5mL of distilled water and stirred for 1hr. 1g of the powdered bones was added and then stirred again for 0.5hrs. This was then incubated in an oven at 60°C for 12 hrs. The caviar hydraulic compressor was used to press the sample at 4 metric tones to give a sample pellet of 2.2mm thickness and 2cm in diameter.

The PHWE Rontgengerat 35kev complete with a rate meter was used as the source of low energy photon. The crystal post was removed and the samples were placed in front of the detector. The X-ray source, sample and detector are aligned in a straight line. The beam kept normal to the surface of the sample to prevent the beam deflection. The readings from digital counter versus thickness was recorded.

The SEM images carried out at the center of nano technology available at our school.

3. Result and discussion
The aim of this study was to compare chemical and structural properties of spongy and hard bone using IR spectroscopy, XRD and SEM. In particular, crystallinity, collagen cross-linking, and carbonate substitution were analyzed. Figure 1 shows IR spectra (400 - 2000 cm⁻¹) of hard and spongy bone. The IR spectral region from 800–1200 cm⁻¹ is dominated by mineral bands whereas the region from 1200–1800 cm⁻¹ contains mainly collagen bands. Carbonate bands are found between 860 and 880 cm⁻¹ with a maximum at 877 cm⁻¹. The IR assignments are included in table 1.
3.1 Curve fitting

Originlab was used to analyze the acquired IR spectra data to reveal the subbands which have been used in this finding. Curve-fitting process of $v_1$, $v_3$ phosphate, amide I envelope and the weak shoulder of carbonate CO$_3^{2-}$ $v_1$. The Gaussian shape was considered and a linear baseline was assumed. Peak finding method based on 2nd derivative and the filtering peak depend on height peak with no smoothing.
The amide I band from 1590 to 1700 cm\(^{-1}\) is assigned to the peptide backbone of proteins. It is composed in IR spectra of seven major subbands that have been correlated with secondary structure motifs. They are located at 1610 cm\(^{-1}\) (aromatic rings of amino acids), 1630 cm\(^{-1}\) (β-sheets), 1645 cm\(^{-1}\) (random coils), 1660 cm\(^{-1}\) (α-helix), 1678 cm\(^{-1}\) (β-sheets), 1690 cm\(^{-1}\) (turns) and a component at 1699 cm\(^{-1}\). Representative curve fitting results are presented in figure 2 A. Collagen cross-links provide the fibrillar collagen matrices with properties like tensile strength and viscoelasticity. Collagen crosslinking is measured as changes in the amide I envelope. The biochemical analysis of collagen model peptides showed that pyridinoline (Pyr) crosslinks resulted in a band at 1660 cm\(^{-1}\) and dehydrodihydroxylysinonorleucine (de-DHLNL) crosslinks in a band at 1690 cm\(^{-1}\). It is known that the content of de-DHLNL crosslinks decreases with bone collagen maturity, while Pyr crosslink content increases. The results are summarized in table 2. The collagen cross links index was found to be approximately twice higher in spongy (cancellous) bone compared to the hard (cortical) bone.

| Collagen cross link | Hard bone | Spongy bone |
|--------------------|-----------|-------------|
|                   | 1.59      | 3.91        |

Table 2: collagen cross-link index in bone obtained from IR spectra.

### 3.3 Carbonate substitutions

The carbonate ν\(_2\) vibration of CO\(_3^{2-}\) in IR spectra at 876 cm\(^{-1}\) was subjected to curve fitting from 850 to 900 cm\(^{-1}\) and revealed differences in the carbonate substitution of spongy and cortical bone (Fig. 2 C and D). The curve-fitting was performed with two components for the cortical bone spectra, while three components were used in spongy spectra. Bands at 871 and 878 cm\(^{-1}\) are related to ν\(_2\) of B-type and A-type CO\(_3^{2-}\), respectively. The band at 860 cm\(^{-1}\) was assigned to ν\(_2\) of A2-type CO\(_3^{2-}\). The appearance of the A2 type substitution could be attributed to the fact that the hydroxyl site can accept two vacancies and the CO\(_3^{2-}\) has consequently more degree of freedom that causes the increase of disorder. The ratios are included in table 3. The values (C/P) are highest for spongy bone. The higher carbonate content in spongy bone is responsible for a higher crystal disorder and is consistent with lower crystallinility according to Table 3.
### 3.4 Crystallinity

The phosphate IR band was fitted under the area from 900 to 1200 cm\(^{-1}\) with seven components near 960, 1020, 1030, 1055 and 1110 cm\(^{-1}\). The band at 960 cm\(^{-1}\) is associated to the \(\nu_1\) symmetric stretching vibration of PO\(_4\)^{3−}, 1020 cm\(^{-1}\) is assigned to HPO\(_4\)\(^{2−}\) in crystalline apatite, 1030 and 1055 cm\(^{-1}\) are related to the \(\nu_3\) vibration of PO\(_4\)^{3−}, and 1070 and 1110 cm\(^{-1}\) to \(\nu_1\) CO\(_3\)\(^{2−}\) at the substitution site B and A, respectively [17]. A representative fitting result was included in Fig. 2 B. The ratio of the subbands 1030 cm\(^{-1}\) to 1020 cm\(^{-1}\) is widely used in the literature to study the mineral crystallinity and has been correlated with the crystal size and perfection as determined by X-ray diffraction line broadening [18]. Crystallinity is a metric related to mineral maturity, and is a measure of mineral crystallite size, mineral maturity, and the amount of substitution into the apatitic lattice. Crystallinity increases when crystals are larger and more perfect (i.e., less substitution), and is directly proportional to the inverse width of the 002 reflection (c-axis reflection) in the powder X-ray diffraction pattern of bone mineral as shown in figure 3. The result summarized on table 4, it is clearly shown that the hard bone is more crystallized than the spongy bone, the IR data and the XRD data confirmed each other.

| Type   | Hard bone | Spongy bone | C/P ratio | C/P ratio |
|--------|-----------|-------------|-----------|-----------|
| A type | 16.4      | 13.2        | 0.076     | 0.086     |
| A2 type| -         | 11.2        | -         | 0.073     |
| B type | 83.6      | 75.6        | 0.263     | 0.303     |

Table 3: Relative amount of A, A2 and B carbonate substitution in hard and spongy bone obtained by curve fitting of IR band near 870 cm\(^{-1}\). The carbonates to phosphate ratios using the subbands of carbonate profile at 870 cm\(^{-1}\) to phosphate at 960 cm\(^{-1}\), were included.

Figure 3: XRD patterns of spongy (black) and hard bone (red).
IR ratio: $I_{1030}/I_{1020}$

|          | IR ratio: $I_{1030}/I_{1020}$ | XRD: FWHM of (002) pattern |
|----------|-------------------------------|-----------------------------|
| Hard bone | 0.523                         | 1.11                        |
| Spongy bone | 0.202                       | 1.76                        |

Table 4: crystallinity in hard and spongy bone obtained from IR and XRD. IR data were calculated from the ratio of subbands 1030 cm$^{-1}$ and 1020 cm$^{-1}$. The XRD data were calculated from the width at half maximum of (002) apatite pattern.

3.5 SEM

The bone microstructures were examined by using a scanning electron microscope (SEM). The hard bone, is made up many rod-like units called osteons or Haversian systems which run longitudinally within the bone. Haversian systems have a central Haversian canal which carries blood and lymphatic vessels and nerve branches.

In figure 4, osteons or Haversian systems can be seen. The central dark circle is the Haversian canal, the canal is surrounded by rings a concentric lamellae of calcified bone matrix. Spongy bone consists of a lattice of thin threads of bone called trabeculae and is less dense than hard bone.

![Figure 4](image)

Figure 4: SEM micrographs of bone tissue: (A) spongy and (B) hard bone.

4. Conclusion

On this investigation we focused on study of structural and chemical features of hard and spongy bone. FTIR, XRD and SEM were used. IR data showed that the collagen cross-links occurred to be higher in spongy bone, and crystallinity was lower in spongy bone. Deconvolution of the infrared band near 870 cm$^{-1}$ reveals evidence for A2-type carbonate substitution on hydroxyapatite of spongy bone in addition to the A and B type carbonate substitution that are also found in hard bone. IR and XRD data confirmed the results of each other. The microstructure was examined by using scanning electron microscope and the result showed that the lattice of thin threads in spongy bone and is less dense than hard bone. These results probably give more information beside the published scientific articles in the literature for more understanding the nature of bone structure to develop next generation of biomaterials and clinical application.
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