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Fourier transform Raman spectroscopic studies of human bone

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We have concluded preliminary investigations concerning the composition of human cortical bone tissue, and of a number of samples of synthetic hydroxyapatite, using Fourier transform Raman (FT-Raman) spectroscopy. Deproteination procedures to remove the bulk of the collagen present in the tissue have allowed isolation of the mineral phase, and a comparison of the spectra obtained from the latter with those of synthetic hydroxyapatites has highlighted a number of discrepancies. FT-Raman spectra obtained for whole, wet bone samples have produced a degree of spectral detail that is much improved upon that available from existing studies. This has mainly been achieved by the successful elimination of fluorescence from the tissue, a problem which has dogged the Raman analysis of bone in particular.

1. Introduction

Cortical bone consists of two primary components; an inorganic or mineral phase, which is mainly a carbonated form of a finely crystalline calcium phosphate, thought to closely resemble hydroxyapatite (HAP), and an organic phase which is made up mainly of Type I collagen fibres [1, 2]. Additional calcium phosphate phases have been proposed as constituents of the mineral phase of bone tissue, such as octacalcium phosphate and amorphous calcium phosphate. Other constituents of bone tissue include water and organic molecules, such as glycosaminoglycans, glycoproteins, lipids and peptides. Ions such as sodium, magnesium, fluoride and citrate are also present, as well as hydrogenophosphate.

Traditional tools used in assessing the chemical composition of bone include light microscopy, electron microscopy, X-ray diffraction and wet chemical analysis. Clearly, in preparation for such analyses, the tissue is subjected to many processes which may alter its structure and composition [3], raising the possibility that any conclusions drawn may not be applicable to the real sample. The ideal analytical tool would be one in which minimal tissue preparation is required, whilst allowing no loss, or even an improvement, of the amount and quality of information derived from the technique.

The techniques of Fourier transform infrared (FTIR) spectroscopy and FT-Raman spectroscopy are increasingly becoming applicable as analytical techniques for biomedical applications, principally because of the following criteria [4]:

(a) The need for tissue preparation is minimal – as an added bonus, the measurement itself is non-destructive to the tissue and very small amounts of material (micrograms to nanograms) can be successfully analysed.

(b) Molecular (ultra structural) level information is available from both techniques, allowing investigation of functional groups, bonding types and molecular conformations.

Prior to the advent of FT-Raman spectrometers, fluorescence was a major problem associated with natural materials since the excitation laser operates in the visible region of the spectrum. Utilizing a near-infrared (Nd:YVO4), such as in the present study, leads to the virtual elimination of fluorescence from most biological samples. Using non-FT-Raman instrumentation, the problem of fluorescence has been dealt with by deproteination of bone samples [5] but clearly, information regarding the organic component is lost.

This study investigated the composition of cortical bone tissue in the fresh state and in the deproteinated state and compared the spectra obtained with those reported by other workers [5, 6]. We have also obtained FT-Raman spectra from a number of synthetic hydroxyapatites. These are compared with spectra obtained from bone and also assessed in the light of previous studies [7, 8].

2. Materials and methods

Cortical bone, obtained from the mid-shaft region of human femoral tissue was utilized in this study. The tissue was obtained in a frozen state.

2.1. Preparation of bone samples

Cubes of cortical bone tissue were prepared, measuring 4 mm along each axis. Four samples were prepared in all from which two were then deproteinated.

The method of preparation involved cutting of the tissue using a small hacksaw and a diamond band saw (the 'EXAKT cutting-grinding system'). The band saw

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used a recirculating water system, which tended to bathe the samples as they were being cut. The time during which the bone was exposed to room temperature (during the preparation process) was kept to a minimum. The samples were refrozen as soon as cutting was completed. They were allowed to thaw for 30 min at room temperature prior to spectrochemical analysis. The transverse faces of the cubes (with respect to the long axis of the femur) were analysed.

Where deproteination was used the procedure of Termine and co-workers was followed [9].

2.2. Instrumentation

A Nicolet 910 FT-Raman spectrometer utilizing a Nd:YVO4 excitation laser operating at 1064 nm was used in this study. Spectral parameters were: 4 cm^{-1} resolution, 300 scans, 650 mW laser power, white light corrected.

3. Results

The most striking feature of the spectra of whole bone tissue obtained from this work is the almost complete absence of fluorescence (Figs 1 and 2), allowing the observation of well-defined peaks.

The deproteination procedure has clearly resulted in a considerable decrease in the scattering intensity of some peaks especially in the regions 2930 and 1443 cm^{-1}, these are the peaks associated with the organic components of bone tissue. Other peaks have remained fairly unaffected, especially the large, narrow

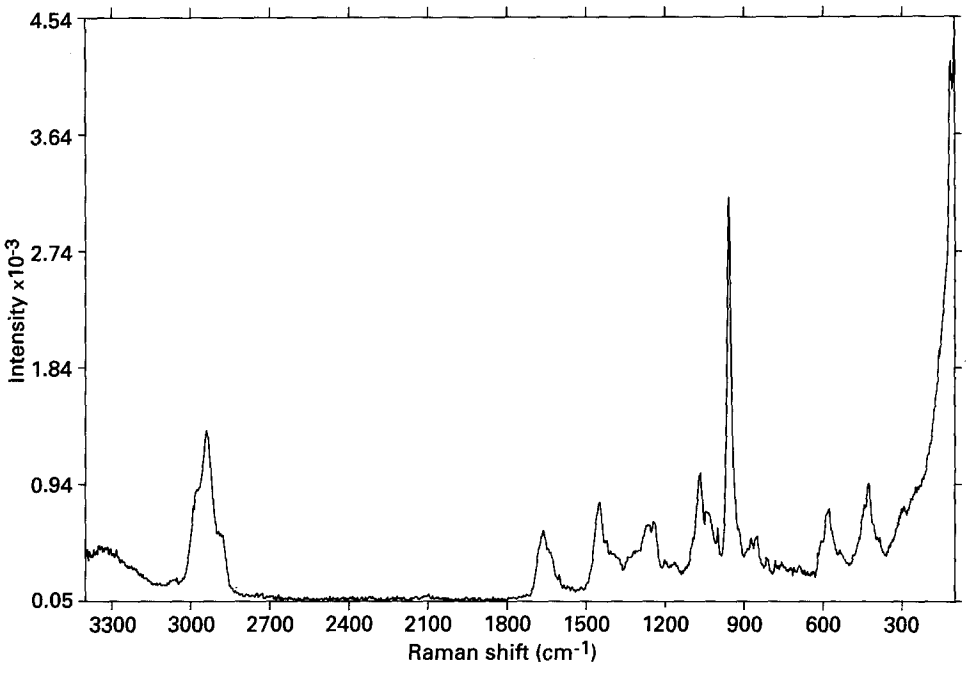

Figure 1 FT-Raman spectrum of whole human bone.

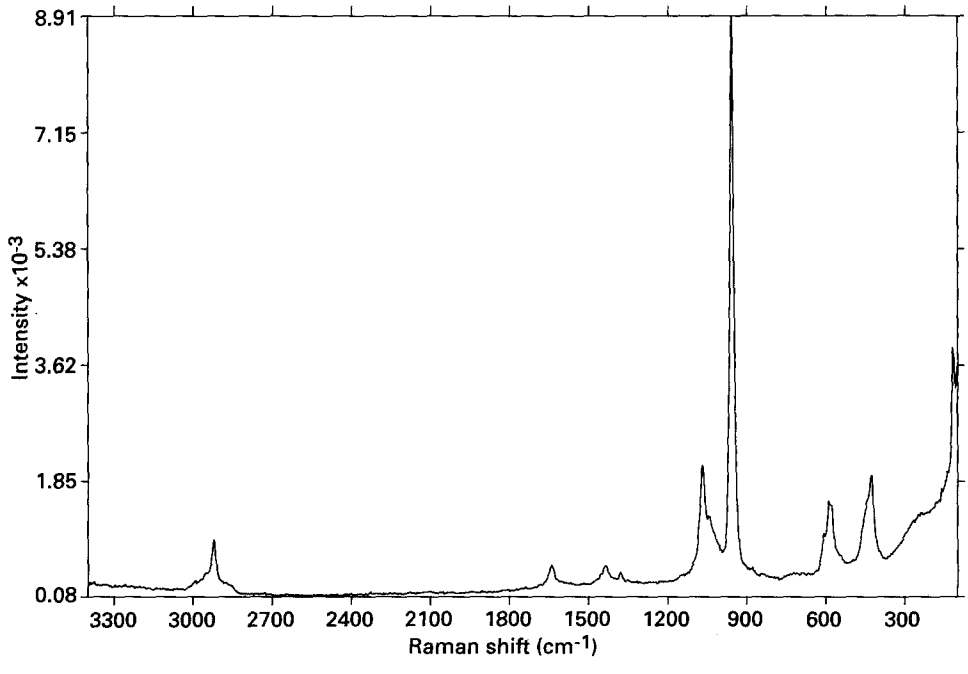

Figure 2 FT-Raman spectrum of deproteinated human bone.

peak at 950 cm^{-1}; these peaks are associated with the mineral component of bone tissue. Peak assignments are given in Table I.

A typical spectrum of synthetic HAP is shown in Fig. 3. The only correlation between the spectrum of bone and synthetic HAP is with the shared peak at 959 cm^{-1}, assigned to the symmetrical phosphate stretching mode. For comparison purposes, spectra of HAP containing carbonate and calcined bone are shown in Figs 5 and 6, respectively.

TABLE I Peak assignments

Peak position (cm^{-1})			Assignment
Human bone		Hydroxyapatite	
Whole	Deproteination		
2940			(C–H) stretch
	2924		(O–H) stretch
1660			Amide I band
1450			(C–H) bend
1270	1070	1070	(C=O) stretch, carbonate
960	960	960	(P=O) stretch, phosphate
		763	unassigned
		694	unassigned
583	583		PO_4^{3-} (bend)
422	422	436	PO_4^{3-} (bend)

4. Discussion

Figs 1 and 2 clearly show the advantages of using FT-Raman spectroscopy as a characterization tool for natural tissue such as bone. Considerable detail can be

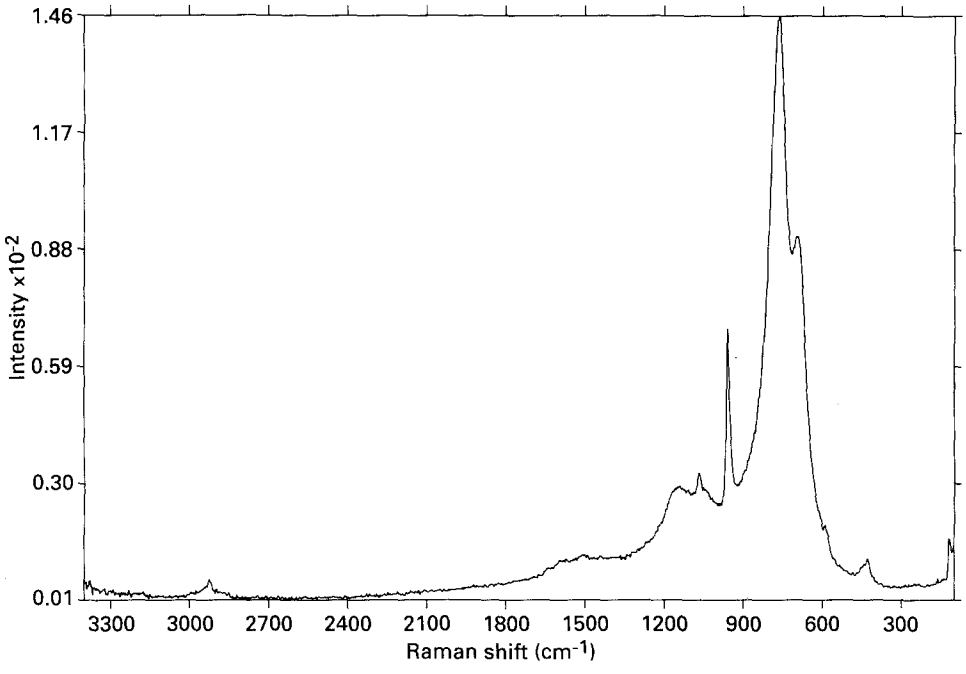

Figure 3 FT-Raman spectrum of synthetic hydroxyapatite.

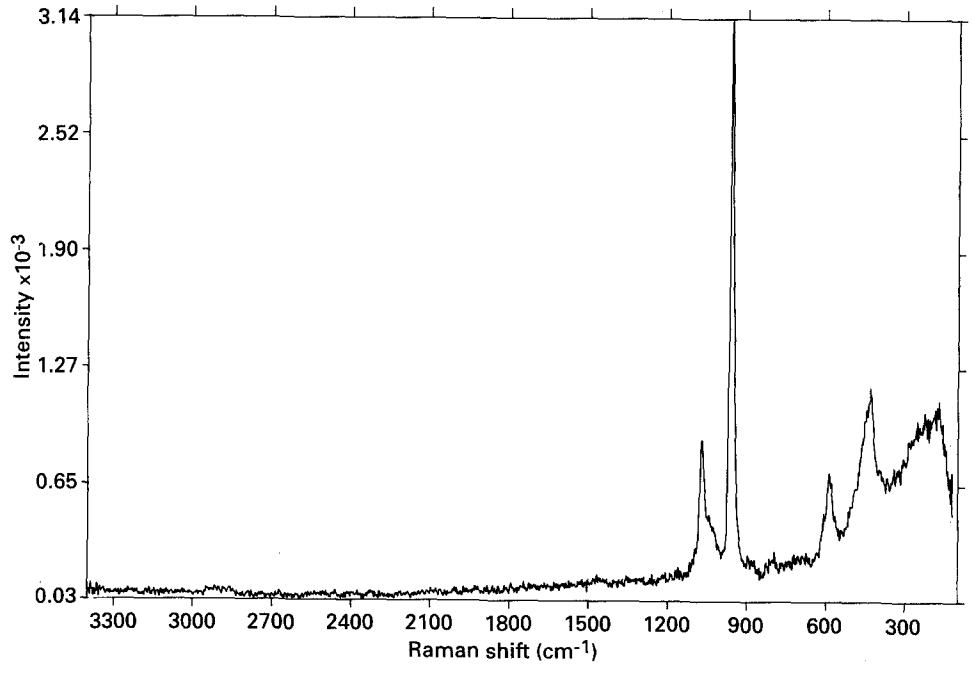

Figure 4 FT-Raman spectrum of synthetic hydroxyapatite containing carbonate.

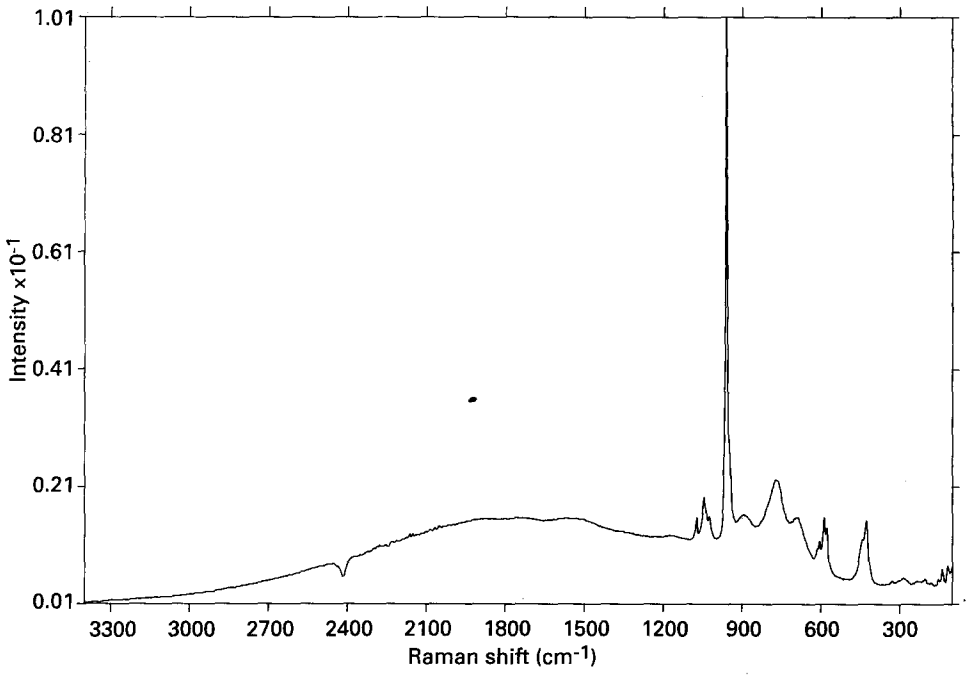

Figure 5 FT-Raman spectrum of calcined bone.

obtained without the need for deproteination. Peaks are well defined and fluorescence is minimal. This is in stark contrast to that observed by Walters *et al.* [5], where deproteination was a necessary step in order to obtain reasonable spectra. Deproteination does improve the signal to noise ratio (Fig. 2) but cannot be justified because of the loss of information concerning the organic components. The spectrum of whole bone (Fig. 1) is identical to that obtained by Gevorkyan *et al.* [6].

The dissimilarity in the spectra of synthetic HAP (Fig. 3) and the mineral component of bone (Fig. 2) is intriguing. The dominant feature in the spectrum of synthetic HAP is a broad doublet at 763 and 700 cm^{-1} which we have been unable to assign. Similar peaks were observed by Tudor *et al.* [10] who assigned them to the hydroxide part of the hydroxyapatite complex. We have been unable to confirm this. We have, however, observed marked changes in the FT-Raman spectrum of HAP when carbonate is incorporated (Fig. 4). This spectrum now closely resembles that of bone mineral (Fig. 2). Substitution into the HAP lattice by carbonate alters both lattice parameters and symmetry which may account for the spectral changes observed. The spectrum of calcined bone (Fig. 5), where the majority of carbonate has been destroyed, shows characteristics from HAP and HAP containing carbonate.

5. Conclusions

Our results from the analysis of bone tissue clearly show the benefits of using FT-Raman instrumentation. Fluorescence has not been a problem during this work, allowing considerable spectral detail to be observed. This study shows that deproteination of bone is not necessary in order to obtain good quality spectra. This is in complete contrast to the situation encountered with conventional Raman analysis, where deproteination of bone tissue is essential.

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