Characterization of Synthetic Hydroxyapatite Fibers Using High-Resolution, Polarized Raman Spectroscopy

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Abstract
In the Raman spectrum of B-type carbonated apatites, the $v_1 \text{CO}_3^{2-}$ mode (at $\sim 1070 \text{cm}^{-1}$) overlaps the $v_3 \text{PO}_4^{3-}$ band. The latter is readily observed where the $\text{CO}_3^{2-}$ content is low (up to $\sim 3 \text{wt\%}$). The $\text{CO}_3^{2-}$ content of bone is considerably higher ($\sim 7–9 \text{wt\%}$). As a result, the $v_3 \text{PO}_4^{3-}$ band becomes completely obscured. The 1000–1100 cm$^{-1}$ spectral range of carbonated apatite is frequently considered a combined $v_3 \text{PO}_4^{3-}$ and $v_1 \text{CO}_3^{2-}$ region. Here, high-resolution polarized Raman spectroscopy (step size of 0.74 ± 0.04 cm$^{-1}$) provides new insights into synthetic hydroxyapatite (HAp) obtained as micrometer-sized fibers. Compared to bone mineral (deproteinized bovine bone), spectral features of HAp fibers are highly resolved. In particular, the $v_3 \text{PO}_4^{3-}$ band resolves into nine distinct sub-components: 1028, 1032, 1040, 1043, 1047, 1053, 1055, 1062, and 1076 cm$^{-1}$. Parameters including full width half-maximum, intensity, area fraction, intensity ratio, and area fraction ratio vary between parallel and perpendicular polarized configurations. It is likely that the $v_1 \text{CO}_3^{2-}$ band of B-type carbonated apatites may contain a small but not insignificant contribution from the 1076 cm$^{-1}$ sub-component of the $v_3 \text{PO}_4^{3-}$ band. Furthermore, the 1076 cm$^{-1}$/1047 cm$^{-1}$ ratio changes between parallel and perpendicular scattering configurations, suggesting that the contribution of the 1076 cm$^{-1}$ sub-component may vary as a function of local orientation of bone mineral, thus skewing the $v_1 \text{CO}_3^{2-}$ band and compromising accurate estimation of carbonate-to-phosphate ratios in B-type $\text{CO}_3^{2-}$ substituted apatite.

Keywords
Hydroxyapatite, calcium phosphate, Raman spectroscopy, biomaterials, bone

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Introduction
Synthetic- and natural-derived calcium phosphates, e.g., hydroxy- or hydroxy(l)apatite (HAp), are by far the most frequently used biomaterials for bone repair. Naturally occurring (geological and biological) apatites can incorporate a plethora of anionic, cationic, and anionic complex substitutions. Likewise, ion substitutions can be easily achieved in synthetic apatites in order to tailor chemical stability or degradation behavior, and bone/biological response. Raman spectroscopy is frequently employed for nondestructive assessment of bone quality and has been used extensively to study carbonate substitution in bone mineral, which is considered an important marker of bone turnover.

In a typical Raman spectrum of B-type carbonated apatites (where $\text{CO}_3^{2-}$ substitutes for $\text{PO}_4^{3-}$), the symmetric stretching $v_1 \text{CO}_3^{2-}$ ($b_{2g}$) mode overlaps the antisymmetric stretching $v_3 \text{PO}_4^{3-}$ ($a_{1g}$) band. The latter is observed up to $\sim 3 \text{wt\%} \text{CO}_3^{2-}$ but tends to be completely enveloped by the $v_1 \text{CO}_3^{2-}$ peak in bone, where the $\text{CO}_3^{2-}$ content is significantly higher ($\sim 7–9 \text{wt\%}$). $\text{CO}_3^{2-}$ substitution for $\text{PO}_4^{3-}$ influences physical properties including crystallite size, solubility, and thermal stability of biological apatites, in effect restricting mineral crystallinity to below that observed for carbonate-free apatites.

Polarized Raman spectroscopy has been used previously to investigate orientation dependence of bone mineral. Attributed to A- and B-type $v_1 \text{CO}_3^{2-}$ bands, previous

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investigations of scattering configuration dependence of the $\nu_2$ PO$_4^{3-}$ mode in carbonate fluorapatite have been unsuccessful in precisely locating the positions of individual sub-components. Using high-resolution polarized Raman spectroscopy, this work investigates micrometer-sized fibers of carbonate-free synthetic HAp and carbonated apatite of bovine cortical bone to better understand the $\nu_3$ PO$_4^{3-}$ and $\nu_1$ CO$_3^{2-}$ overlap in B-type CO$_3^{2-}$ substituted apatite such as bone mineral in order to enable more accurate estimation and interpretation of carbonate-to-phosphate ratios.

Materials and Methods

Synthetic HAp Fibers and Bovine Bone Mineral

The HAp fibers were obtained by dispersing 5–80 nm apatite particles in an aqueous solution of 200 kDa pullulan (Sigma-Aldrich). This solution was extruded at 1.4 kg/cm$^2$ pressure while simultaneously discharging air at 250 m/s to form a stream of fibers. Using a far-infrared heater under the extrusion nozzles, the fiber stream was heated at 400 °C and blown onto a screen conveyor belt to produce a non-woven fabric that was further heated at a rate of 50 °C per hour and calcined at 1100 °C for one hour. Commerically sourced (https://boneslices.com) bovine cortical bone stored in 96% ethanol was deproteinized using 5% sodium hypochlorite (Honeywell Fluka) for 16 h at 4 °C in order to isolate the inorganic/mineral phase.

Scanning Electron Microscopy and Micro-Raman Spectroscopy

The HAp fibers were visualized using scanning electron microscopy (Ultra 55 FEG SEM, Leo Electron Microscopy Ltd, UK) in the secondary electron mode. Micro-Raman spectroscopy was performed using a confocal Raman microscope (Renishaw inVia Qontor) equipped with a 633 nm laser and LiveTrack focus-tracking technology. The microscope (Ultra 55 FEG SEM, Leo Electron Microscopy Ltd, UK) in the secondary electron mode. Micro-Raman spectroscopy was performed using a confocal Raman microscope (Renishaw inVia Qontor) equipped with a 633 nm laser and LiveTrack focus-tracking technology. The laser was focused down on to the sample surface using a x 100 (0.9 NA) objective. In the 390–1100 cm$^{-1}$ spectral range (step size of 0.81 cm$^{-1}$ at 390 cm$^{-1}$ and 0.68 cm$^{-1}$ at 1100 cm$^{-1}$), the Raman scattered light was collected using a Peltier-cooled charge-coupled device deep depletion near-infrared-enhanced detector behind a 2400 g mm$^{-1}$ grating, 10 s integration time, and 10 accumulations. Using a half-wave plate and polarization analyzer, spectra were collected in parallel z(xx)z and perpendicular z(xy)z polarized configurations from $3 \times 3$ point grid-matrices ($n = 8$) for HAp fibers and from $9 \times 8$ point grid-matrices ($n = 1$) for deproteinized bovine bone. The laser power at the sample was ~15 mW. Background subtraction and cosmic ray removal were performed in Renishaw WiRE S software. Spectra from each grid-matrix were averaged and normalized to the 428 cm$^{-1}$ $\nu_2$ PO$_4^{3-}$ sub-component. For HAp fibers, one Lorentzian curve was fitted to the $\nu_1$ PO$_4^{3-}$ peak (~962 ± 10 cm$^{-1}$). Centered at inflection points in calculated second-derivative spectra, $\nu_3$ PO$_4^{3-}$ band (1020–1090 cm$^{-1}$) sub-components were resolved by fitting multiple Lorentzian curves ($r^2 > 0.99$). For each sub-component, the full width half-maximum (FWHM), intensity, and area fraction of the fitted range were obtained. Polarization configuration dependent variation in intensity ratios and area fraction ratios between $\nu_3$ PO$_4^{3-}$ sub-components were expressed as percentage difference values.

Statistical Analysis

For statistical analysis, the non-parametric Wilcoxon signed-rank test was used (SPSS Statistics, v.25, IBM Corporation) and $p$ values < 0.05 were considered statistically significant. Mean values ± standard deviations are presented.

Results and Discussion

The HAp fibers were 3–15 μm thick (Fig. 1a) and carbonate-free (Fig. 1b), since characteristic Raman peaks attributable to CO$_3^{2-}$ ions in calcium phosphates or calcium carbonates were not detected. The symmetric stretching mode, $\nu_1$ PO$_4^{3-}$, was narrow (FWHM = 3.25 cm$^{-1}$) and decreased in intensity by 68 ± 10% ($p = 0.012$) between polarization configurations, i.e., depolarization ratio of 0.32. The $\nu_2$ PO$_4^{3-}$ band consists of sub-components at 428 cm$^{-1}$ and 450 cm$^{-1}$, of which the ~428 cm$^{-1}$ sub-component appears least sensitive to polarization in terms of position, intensity, and shape. The $\nu_4$ PO$_4^{3-}$ band is comprised of sub-components at 579–580 cm$^{-1}$, 590 cm$^{-1}$ with a shoulder at 587 cm$^{-1}$, 607 cm$^{-1}$, and 614 cm$^{-1}$. In comparison, the inorganic phase of bovine cortical bone exhibits a broader $\nu_1$ PO$_4^{3-}$ peak (FWHM = ~17 cm$^{-1}$), attributable to the poor crystallinity of bone mineral, and a strong $\nu_3$ CO$_3^{2-}$ band at ~1072 cm$^{-1}$. The $\nu_2$ CO$_3^{2-}/\nu_1$ PO$_4^{3-}$ intensity ratio (~0.18) does to change between polarization configurations. In addition, a wide shoulder is present in the 1025–1055 cm$^{-1}$ range corresponding to the $\nu_3$ PO$_4^{3-}$ band. The individual sub-components of the $\nu_2$ PO$_4^{3-}$ and $\nu_4$ PO$_4^{3-}$ bands are poorly resolved.

The profile of the $\nu_3$ PO$_4^{3-}$ mode of synthetic HAp fibers varies between scattering configurations. Second-derivative spectra reveal nine distinct inflection points in the 1020–1090 cm$^{-1}$ range (Fig. 2). Previously, Awonusi et al. have predicted six sub-components at 1029, 1040, 1047, 1053, 1062, and 1076 cm$^{-1}$. However, each of the features at 1029, 1040, and 1053 cm$^{-1}$ are found to resolve into two sub-components: 1028 cm$^{-1}$ and 1032 cm$^{-1}$, 1040 cm$^{-1}$ and 1043 cm$^{-1}$, and 1053 cm$^{-1}$ and 1055 cm$^{-1}$, respectively.
which alters the intensity ratios and area fraction ratios between individual sub-components. It is, therefore, demonstrated that \(n^3\) PO\(_4^{3–}\) band profile/shape is not conserved between polarization configurations and that the individual sub-components exhibit dissimilar levels of polarization dependency. Of particular significance is the relationship between 1076 cm\(^{-1}\) and 1047 cm\(^{-1}\) sub-components, which are two of the strongest features of the \(n^3\) PO\(_4^{3–}\) band. Attributable to increases in 1076 cm\(^{-1}\) sub-component intensity and area fraction, the 1076 cm\(^{-1}\)/1047 cm\(^{-1}\) ratios differ by \(\sim 68–69\% \ (p = 0.012)\) between the polarization configurations (Fig. 4).

Biological apatites contain varying amounts of CO\(_3^{2–}\). The CO\(_3^{2–}\) content of bone is taken as an indicator of bone maturation and turnover,\(^7,17\) and considerable attention is given to the carbonate-to-phosphate ratios of bone affected by compromised systemic conditions\(^18–20\) and bone surrounding implant biomaterials.\(^21,22\) It is likely that the 1076 cm\(^{-1}\) sub-component of the \(n^3\) PO\(_4^{3–}\) band contributes to the \(v_1\) CO\(_3^{2–}\) band, but remains a challenge to
deconvolute. The scattering configuration dependency of
the 1076 cm\(^{-1}\)/1047 cm\(^{-1}\) ratio is also noteworthy. The con-
tribution/intensity of the 1076 cm\(^{-1}\) sub-component may
vary as a function of local, sub-micrometer/nanoscale orien-
tation of bone mineral, thereby skewing the shape, intensity,
and/or integral area of the \(\nu_3\) CO\(_3^{2-}\) band, thereby compro-
mising the accuracy of carbonate-to-phosphate ratios in B-
type CO\(_3^{2-}\) substituted apatite.

Conclusion

The \(\nu_3\) PO\(_4^{3-}\) band in carbonate-free HAp fibers resolves
into at least nine sub-components in the 1020–1090 cm\(^{-1}\)
spectral range. Sub-component FWHM, intensities, area
fractions, and intensity and area fraction ratios vary
between \(z(xx)z\) and \(z(xy)z\) configurations. The sub-compo-
ments at 1028, 1032, 1047, 1055, and 1076 cm\(^{-1}\) exhibit
pronounced sensitivity to the polarization configuration.
A noteworthy change is in the 1076 cm\(^{-1}\)/1047 cm\(^{-1}\) ratio
between parallel and perpendicular polarized configurations.
It is likely that the 1000–1100 cm\(^{-1}\) range of B-
type carbonated apatites, containing the \(\nu_1\) CO\(_3^{2-}\) band,
also includes a small contribution of the 1076 cm\(^{-1}\) sub-
component of the \(\nu_3\) PO\(_4^{3-}\) band, which may vary as a func-
tion of local, sub-micrometer/nanoscale orientation of bone
mineral, thereby skewing the shape, intensity, and/or inte-
gral area of the \(\nu_1\) CO\(_3^{2-}\) band, thereby compromising the
accuracy of carbonate-to-phosphate ratios in B-type CO\(_3^{2-}\)

Figure 3. Polarization affects \(\nu_3\) PO\(_4^{3-}\) sub-component (a) FWHM, (b) intensity, and (c) area fraction. Wilcoxon signed-rank test
\((n = 8)\). Asterisk (*) denotes statistically significant difference between \(z(xx)z\) and \(z(xy)z\) configurations.

Figure 4. Polarization affects \(\nu_3\) PO\(_4^{3-}\) sub-component (a) intensity ratio and (b) area fraction ratios. Wilcoxon signed-rank test
\((n = 8)\). Asterisk (*) denotes statistically significant difference between \(z(xx)z\) and \(z(xy)z\) configurations.
substituted apatite. For this reason, carbonate-to-phosphate ratios determined using Raman spectroscopy must be interpreted with caution.

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Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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