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Chemical composition and structure of peritubular and intertubular human dentin revisited

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Abstract

Objective—Currently there is still a debate about whether peritubular dentin (PTD) is non-collagenous or collagenous tissue. The chemical composition and structure of human PTD and intertubular dentin (ITD) was re-visited in this study.

Design—The dentin tubular region including ITD and PTD prepared from human third molars was *in-situ* detected by means of micro-Raman spectroscopy (μ Rs) and atomic force microscopy (AFM).

Results—From the μ Rs study, it was found that the mineral/matrix ratios (phosphate vs CH_2) in PTD were ~ 3 times of those in ITD. For the mineral, the differences between PTD and ITD were small, but still detectable. For the organic matrix, the intensity ratios of amide III to CH_2 in ITD were ~ 1.5 times of those in PTD, indicating the structural differences. In addition, there was a higher proline/hydroxyproline content in ITD than that in PTD. However, the overall Raman peak contour in the amide regions (I & III) was similar, indicating collagen might still exist in both the ITD and PTD. An *in situ* AFM observation of the dentinal tubular region during EDTA etching confirmed that dentin collagen ran across from the ITD into the PTD.

Conclusion—A phenomenon similar to that observed in the dentin-enamel junction is proposed to explain the above results. It is demonstrated that the μ Rs-AFM approach can be used to provide an insight into the structure of small dental tissues at the micron or sub-micron scale.

Keywords

Raman microspectroscopy; peritubular dentin; intertubular dentin; atomic force microscopy (AFM)

1. Introduction

Mature vertebrate tooth dentin is a hard, elastic, avascular mineralized tissue supporting enamel and enclosing the central pulp chamber. It is composed of approximately 70% mineral, 20% organic matrix and 10% water by weight, and 45%, 33%, and 22% by volume, respectively.[1] The mineral phase in dentin mainly consists of carbonate-substituted hydroxylapatite ($\text{Ca}_{10}(\text{PO}_4)_{6-x}(\text{OH})_{2-y}(\text{CO}_3)_{x+y}$, where $0 \leq x \leq 6$, $0 \leq y \leq 2$) in a form of

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small plates. Furthermore, the organic phase contains about 90% fibrous proteins (primarily type I collagen), while the rest of the organic phase is comprised of lipids and non-collagenous matrix proteins. [1] It is reported that the growth of apatite's crystallites occurs with the long axis running parallel to the long axis of collagen fibrils,[2] and their nucleation is associated with non-collagenous proteins, *e.g.*, phosphophoryn.[3]

When viewed under optical and scanning electron microscopes, the peritubular dentin (PTD) in transverse cross-section [4, 5] appears as ring-like structures. The rest of the tissue around the dentinal tubules is called intertubular dentin (ITD). In a tooth, it is shown that the relative area occupied by PTD and ITD as well as the numerical density of dentinal tubules vary significantly from the dentin-enamel junction (DEJ) to mineralization front (MF).[6] For example, the PTD only occupies ~3% relative area at the DEJ, but increases to >60% at the MF. Currently, available information on chemical composition and structure of dentin as described above is mainly referred to the ITD. Due to the small size of the PTD and its adjacent connection with the ITD, the chemical composition and structure of this type of dentin are not well implicit.

In terms of the mineral phase of the PTD, there have been valuable studies on elemental composition, crystal structure and orientation and some physical properties.[7] Thus far, it is known that the PTD is a highly calcified or hypermineralized tissue. Miller[8] and Höhling[9] respectively reported 9% and 40% compositional differences in the content of elements (such as Ca, P and Mg) between the ITD and PTD using electron probe analyses. Moreover, using X-ray microanalysis, Yoshiyama et al[10] found that the Ca/P ratio in the PTD was 1.49, which was matching with that in ITD. With regards to crystal structures, Lester and Boyde[11] have observed that the PTD is comprised of small, *ca.* 250 Å, equal-sided micro-crystals while the ITD is characterized by typical larger hexagonal crystalline plates. Schroeder and Frank[12] have reported that the micro-crystals in the PTD do not show a preferred crystal orientation as in the ITD where the *c*-axes run parallel to the collagen fiber axes. In view of physical properties, Marshall [13] et al have reported that the PTD shows a uniform Young's modulus but the Young's modulus changes as a function of location in ITD. In an earlier study, it is [14] indicated that the crystals in PTD are difficult to be resolved because they have an extremely compact organization. On the other hand, some researchers have recently reported that the PTD dissolves more easily in acid than the ITD.[4, 14]

Compared to the mineral phase, less is acknowledged on the organic matrix phase in the PTD. It is known that there is less organic matter existing in the PTD than in the ITD. However, whether the organic matrix contains collagen or other non-collagenous proteins (*e.g.* phosphoproteins) has been a focus of debate. Early results [15–18] observed by electron microscopy (including SEM and TEM) showed there were collagen fibers in the PTD but much less than those in the ITD. However, other researchers recently reported that the PTD did not contain collagen but only phosphoproteins. [19] [20, 21] Additionally, it was [22] reported that the PTD was a non-collagenous tissue because of no detectable piezoelectric response in this domain, since piezoelectric response was considered as characteristics of collagen. However, Magne *et.al.* [23] concluded that collagen did exist in the PTD when they analyzed the horse PTD structure with Fourier transform infrared microspectroscopy (FTIRM). In the current study, the chemical composition and microstructure of PTD was revisited by using Raman microspectroscopy and *in-situ* atomic force microscopy (AFM), respectively.

2. Materials and Methods

2.1 Preparation of dentin specimens

Five young adult human third molars were collected according to the protocol approved by the University of Missouri Kansas City health sciences institutional review board (IRB). These molars were stored in 0.9% phosphate buffer saline (PBS) buffer solution (pH=7.4) containing 0.002% sodium azide at 4°C. A slow-speed water cooled diamond saw (Buehler Ltd., Lake Bluff, IL, USA) was used to remove the roots. The remaining tooth specimens were sectioned into beams of 2.5 mm × 3.0 mm × 6 mm along the long axis of the teeth. To avoid the dentinal tubules to be covered by smear layer,[24] the dentin beams were not cut, but mechanically fractured with exposed tubules perpendicular to the fractured surfaces. The detailed process was as follows: two notches with the depth of ~0.3 mm were first made in the two opposite sides of a dentin beam close to the crown side, to ensure fracture occurs at the superficial dentin region. The beams were placed into liquid nitrogen for 10 min, then fractured along the notches. The fractured surfaces with exposed tubules were used to acquire Raman spectra and mapping. The specimens for AFM observations were obtained by fracturing dentin in liquid nitrogen along the direction where the exposed surfaces parallel to dentinal tubules.

2.2 SEM analysis

The fractured specimens described above were prepared for SEM examination. After dehydration, the specimens were mounted on aluminum stubs and sputter coated with ~20 nm of gold-palladium. The specimens were then examined at a variety of magnifications in a Philips XL30 ESEM-FEG (Philips Electron Optics Inc., Hillsboro, OR) at 10 kV.

2.3 Micro-Raman spectroscopic analysis

To accurately investigate the chemical composition of the ITD and PTD, a LabRam HR 800 Raman spectrometer (HORIBA JOBIN YVON, Longjumeau, Paris, France) using monochromatic radiation emitted by a He-Ne laser (632.8 nm) was used to collect Raman spectra and mapping. It was equipped with a confocal microscope (Olympus BX41), a piezoelectric XYZ stage with a minimum step width of 50 nm, and an air-cooled CCD detector of 1024×256 pixels. The following parameters were used: 600 grating, 140-μm confocal hole, 100-μm slit width, operating at excitation power of 20 mW. Spectra were Raman-shift-frequency-calibrated using known lines of silicon.

Micro-Raman spectra were acquired by focusing the laser beam through a 100× Olympus objective to a ~0.8–0.9 μm beam diameter onto the fractured dentin specimens. Spectral mapping was performed at positions corresponding to 1-μm intervals across the dentin surfaces containing some tubules using the computer-controlled stage. Two consecutive scans of spectra (with 60 seconds accumulation time each) were obtained from each site. An imaging system and high-resolution monitor enabled visual identification of the position at which the Raman spectra were obtained. The individual spectra of PTD were acquired from intratubular region or inner edge of tubules. To ensure the Raman signal only from the PTD, we placed the laser beam with a diameter of ~0.8–0.9 μm in the center of a tubule whose diameter was ~1 μm. The spectra of ITD were collected from the middle region between dentinal tubules. The doubled accumulation time was used for the individual spectra to make sure high signal to noise ratios. Labspec 5 software (HORIBA JOBIN YVON) was used to analyze the acquired Raman spectra and mapping data. A multi-point baseline technique was used to adjust those individual spectra, and a polynomial baseline technique was used to adjust the Raman mapping. Raman images were obtained based on the peak intensities of amide I at 1670 cm⁻¹ or ν₁ vibration of phosphate at 961 cm⁻¹ using the Labspec 5.

2.4 In-situ AFM analysis

The region surrounding dentin tubules obtained by frozen fracture was observed *in-situ* using a Picoplus AFM system (Molecular Imaging, Tempe, Az, USA) in a contact mode during etching in the ethylenediaminetetraacetic acid / 2,2',2'',2'''-(ethane-1,2-diyl)dinitrilo)tetra acetic acid (EDTA) solution (0.02 M, pH 7.4) for 5 min. Oxide sharpened Si_3N_4 cantilevers having a nominal force constant of 0.08 N/m (OMCLTR400PS) were purchased from Olympus Ltd (Tokyo, Japan) and used in this study. A typical scan speed was set at 1.2 lines/s, and force setpoint was -2.240V . Three data channels, topography, deflection and friction, were acquired simultaneously. The 5.3.3 USB Picoscan software was used to control the instrument and display the obtained images.

3. Results

Typical SEM image from fractured dentin surface with dentinal tubules which are perpendicular to the surface is shown in Fig. 1. The mean diameter of dentin tubules is about $1.21 \pm 0.08 \mu\text{m}$ and the average width of the PTD is about $0.80 \pm 0.09 \mu\text{m}$. The distance between two tubules is in a range of $5 \mu\text{m}$ to $10 \mu\text{m}$ as observed in Fig. 1. The backscattered SEM image corresponding to Fig. 1 (data not shown) shows that the PTD region is brighter than the ITD region, indicating higher mineralization of the PTD than the ITD.

Representative Raman spectra obtained from the ITD (black bold line) and PTD (red dash line) are shown in Fig. 2. Most of Raman peaks, which are related to inorganic mineral and organic matrix, are within the range of $400\text{--}1800 \text{ cm}^{-1}$. The identification and assignment of the Raman sensitive peaks associated with organic and inorganic components of dentin is well documented. Some of the important and relevant assignments in this region are as follows; the peaks associated with mineral appeared in the spectral range from 1010 to 1100 cm^{-1} , 928 to 984 cm^{-1} and from 400 to 650 cm^{-1} , including ν_1 vibration of B-type carbonate, ν_3 , ν_1 , ν_2 , ν_4 vibration peaks of phosphate group. [25] The major peaks associated with organic component appeared in the spectral range from 1580 to 1720 cm^{-1} , 1435 to 1492 cm^{-1} , and from 1217 to 1311 cm^{-1} , containing the amide I, CH_2 deformation and the doublet peaks of amide III, respectively. The detailed information about assignments and positions of these peaks is listed in Table 1. [25–28]

The intensities of the mineral peaks in the spectrum of PTD were much stronger than those of ITD. The magnified spectrum showing these mineral peaks in the range from 350 to 1150 cm^{-1} was inserted in the Fig. 2. All the spectra were normalized based on the intensity of the CH_2 deformation peak at 1450 cm^{-1} . The ratios of the mineral carbonate peak at 1071 cm^{-1} and the phosphate peak at 961 cm^{-1} to the CH_2 peak (1450 cm^{-1}), respectively, are shown in Table 2. These ratios in the PTD were 2.8–3 times higher than those in the ITD.

After subtracting the organic component from Raman spectra of the ITD and PTD, the regions from 900 to 990 cm^{-1} and from 1010 cm^{-1} to 1120 cm^{-1} of the difference spectra obtained are shown in Fig. 3. The peak in the range from 900 to 990 cm^{-1} is associated with ν_1 vibration of PO_4^{3-} . Usually the full width at half maximum (FWHM) of this peak is used to measure the degree of mineral crystallinity. The FWHM of this peak was $\sim 17.5 \text{ cm}^{-1}$ for both the ITD and PTD spectra, indicating no difference in crystallinity between PTD and ITD. All spectra were then normalized based on the intensity of this phosphate peak. The peak appearing in the range from 1055 to 1090 cm^{-1} is associated with B-type carbonate, and the peak appearing in the range from 1040 to 1060 cm^{-1} is related to the phosphate in apatite crystals. The peak at 1103 cm^{-1} is associated with A-type carbonate in mineral. The changes in these mineral-derived peaks are not dramatic between the PTD and ITD. The intensity ratios of these peaks are listed in Table 3, which are slightly higher in the ITD than those in the PTD.

Representative Raman spectra in the range from 1200 to 1750 cm^{-1} and from 820 to 900 cm^{-1} for the PTD and ITD are shown in Fig. 4. All spectra were normalized based on the intensity of the CH_2 peak at 1450 cm^{-1} . The peaks in these two ranges are mainly associated with the organic matrix of dentin. Specifically, the peaks in the range of 1200–1750 cm^{-1} are related to amides I, III, and secondary structure of collagen. The changes in these protein-derived peaks are clearly noticeable. For example, in the PTD spectrum, the peaks in the regions of 1550–1600 cm^{-1} and 1300–1350 cm^{-1} increased; however, the intensities of the peaks in the region of 1200–1300 cm^{-1} decreased in the spectrum of PTD. The results indicated that the molecular/secondary structure of organic matrix in the PTD was different from that in the ITD.

The peaks in the range of 820–900 cm^{-1} are related to amino acids in dentin collagen. Specifically, the peak at 850–860 cm^{-1} is associated with proline and the peak at 870–880 cm^{-1} is associated with hydroxyproline in the organic matrix. [29, 30] In the ITD the two peaks appeared at 854 and 875 cm^{-1} ; however they appeared at 853 and 880 cm^{-1} in the PTD, respectively. The intensities of both peaks in the ITD are higher than those in the PTD (Fig. 4), indicating more proline/hydroxyproline content in the ITD. The intensity ratios of the selected peaks in these regions to the peak at 1450 cm^{-1} of ITD and PTD are listed in Table 2.

Although there are differences in the spectra of the PTD and ITD as indicated above, these differences in both mineral- and protein-derived peaks are not dramatic. In order to confirm if there are collagen fibrils in the PTD, AFM was used for the micro-structural analysis. The *in-situ* AFM topography and deflection images of the same dentin specimen containing parallel tubules, PTD and ITD before and after the EDTA etching are shown in Figs. 5, 6. The diameter of dentinal tubules was about 1–1.5 μm , which is consistent with the SEM observation. In Figs. 5 and 6, C and D are the magnified images of the marked areas in A and B, respectively. The more detailed structures are observed in the high magnification images. The images in Fig. 6 show the morphology taken after 5 minutes of etching by 0.01 M EDTA. The diameter of the dentinal tubule shows an increase as compared with that before EDTA etching. A region/gap between the PTD and ITD, which was more susceptible to etching, was found as marked by black arrows in the images after etching by EDTA. However, no gaps were found prior to the EDTA etching. Interestingly, collagen fibrils were found running across this gap, which extended from the ITD into the PTD.

4. Discussion

As a spatial neighbor of the PTD, the ITD constantly becomes an exclusive reference when PTD's structure, composition, and properties are investigated. To date, there has been minimal chemical information related to the PTD as a result of its small size. In the present study, we have used the micro-Raman spectroscopic (μRS) technique to examine the chemical nature of the ITD and PTD including inorganic and organic phases. As compared to the ITD, alterations in chemical structure and composition of the PTD were explored and revealed, which have never been reported previously.

The μRS results showed that the PTD is much more mineralized (or hypermineralized) than the ITD. After normalization based on the CH_2 peak at 1450 cm^{-1} , the intensities of all the peaks related to inorganic phase in the PTD were higher than those in the ITD as shown in Fig. 2. For example, the ratios of the mineral phosphate peak at 961 cm^{-1} to the matrix CH_2 peak at 1450 cm^{-1} were ~ 12.9 for the ITD, and ~ 39.2 for the PTD. The mineral/matrix ratios in the PTD were ~ 3 times higher than those in the ITD. In terms of the components, the PTD and ITD are all composed of hydroxyapatite and matrix proteins. Therefore, the difference of ~ 3 times indicates the content of organic matrix in the PTD is $\sim 1/3$ of that in

the ITD. In a previous study, a series of mixtures with known, different hydroxyapatite levels were used to generate FT-Raman spectra and calibration curves. [31] The relationship between the relative ν_1 phosphate peak intensities and the mineral apatite contents were generated to measure the mineral content in bone. Using the calibration curve generated in that study, the inorganic mineral and organic matrix contents were calculated in the present work. The calculated values for the mineral content in the ITD and PTD were 88% and 96%, respectively, which means the organic contents in the ITD and PTD were 12% and 4%, respectively. Obviously, the resulted values for the organic content are relatively lower. The reason might be due to different operation/instrumental conditions between our instrument (micro-Raman spectroscopy) and their system (FT-Raman spectroscopy). It is known that the organic matrix content in ITD is ~20%, and the inorganic content is ~70%. [32] Accord to the above difference in the mineral/matrix ratio, the organic content in PTD is calculated to be ~7–8%.

In terms of the mineral phase of the PTD, our micro-Raman results revealed that the position, shape, and width of phosphate peak at 961 cm^{-1} were similar to those for ITD, indicating the mineral crystallinity within the PTD is not much different from the mineral in the ITD. There were some minor changes in the carbonate- and phosphate-derived peaks within the spectra range from 1010 to 1120 cm^{-1} (Fig. 3). For example, the intensity ratios of both the peaks at 1071 cm^{-1} (B-type carbonate) and 1103 cm^{-1} (A-type carbonate) to 961 cm^{-1} increased from PTD to ITD, indicating slightly higher carbonate content in ITD. However, overall the differences in these mineral-derived peaks are not dramatic between the PTD and ITD.

In terms of the organic matrix variations, the spectral region of amides I and III was thought to be the best possible region for studying the protein structural changes [24, 29]. The position and intensity of these amide bands are sensitive to the molecular conformation/structure of the polypeptide chains [33] of collagen/proteins. In a comparison of the amides region in the spectra of PTD and ITD (Fig. 4), it could be seen that the amide I peak was broaden, and the amide III peak decreased in the PTD spectrum. The broadening of amide I peak was associated with the protein secondary structure changes. [24] It is known that the amide III peaks in a vibrational spectrum originate from the stretching vibration of C–N [34] (appearing at 1243 cm^{-1}) and plane deformation vibration of N–H [34, 35] (appearing at 1267 cm^{-1}) in the peptide chain of collagen/proteins. Since all spectra have been normalized based on CH_2 at 1450 cm^{-1} , reduced intensity of amide III in PTD indicates either some molecular structural or compositional differences in the peptide chain between ITD and PTD. The differences are further indicated by changes in intensity of the proline peak at 854 cm^{-1} and the hydroxyproline peak at 875 cm^{-1} . Both peaks decreased in the spectrum of PTD, pointing to lower proline/hydroxyproline content in the PTD.

The above results imply that there are structural and compositional differences in the organic matrix between the ITD and PTD. However, when comparing the spectra of the ITD and PTD, it was realized that the spectral contours of amides I and III were similar, which were typical contours for a spectrum of collagen. Whether PTD is a non-collagenous tissue or contains collagen has been of interest to many researchers. The above similarity in our Raman spectra was observed previously by Magne *et.al.* [23] in their FTIR spectra. They concluded that PTD did contain collagen fibers, which were also observed by several groups using SEM or TEM. [15–18] However, in recent studies, it has been reported that PTD is a non-collagenous tissue, mainly contains phosphoproteins. [19–22] One of the main reasons for the debate probably is due to the small size of PTD. The width of the PTD itself is reported to be only ~1 μm while the width of the peritubular-intertubular junction (PIJ) is in a range from 0.5 to 1.0 μm . [36]

The above debate has led us to compare the PTD/ITD/PIJ with the dentin/enamel and dentin-enamel junction (DEJ). It is well known that enamel is a non-collagenous tissue, unlike from dentin. The chemical composition and structure of DEJ has been studied recently by using micro-Raman. [37] Interestingly, it is noticed that the spectral differences between dentin and DEJ, including the broadening of amide I and reduced intensity of amide III, are similar to the differences between PTD and ITD as described above. [37] In addition, the spectral contours of amides I and III are also similar between the DEJ and dentin. It is known that formation of the DEJ begins at the early stages of tooth morphogenesis and is thought to be linked to a mixture of dentin proteins secreted by odontoblasts and enamel proteins from ameloblasts [38–40]. The proteins associated with DEJ formation and collagen fibril bundles that cross the transition zone and insert into enamel [41] might be responsible for the variation and similarity between the spectra of the DEJ and dentin. We suspect that this phenomenon (collagen fibrils running across from ITD to PTD) might also occur in the junction between PTD and ITD during the formation of PTD (a mineral deposition process). Our *in-situ* AFM observation confirms the phenomenon (Fig. 6), which is also supported by the Raman results.

In summary, micro-Raman spectroscopy equipped with confocal microscope and AFM proved to be useful in investigating the chemical and structural information of the PTD/ITD at the small length scales. The advantages of this technique include minimal sample preparation, *in-situ* detection/observation and high spatial resolution. The results show that the PTD is much more mineralized than the ITD, in which the mineral/matrix content/ratios are ~2 times higher than those in the ITD. There are detectable differences in both inorganic and organic phases between the ITD and PTD. Although our results indicate that there is collagen in the PTD, PTD is still likely to be a tissue different from ITD as enamel is different from dentin. A phenomenon or mechanism similar to the enamel/dentin/DEJ is proposed to explain the results of this study as well as the results from previous studies.

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Fig 1. Representative SEM image of fractured dentin surface showing the ITD and PTD. Tubules are perpendicular to the fractured surface. The comparatively compact and flat look of the PTD is observed.

Fig 2.

Representative Raman spectra of the ITD and PTD. The spectra were normalized based on the peak intensity of CH₂ bending vibration at ~1450 cm⁻¹. The inserted figure shows the magnified, same spectra in the range from 350 to 1200 cm⁻¹.

Fig 3.

Representative Raman spectra of the ITD and PTD in the range from 900 to 1120 cm⁻¹. The spectra were normalized based on the peak intensity of ν_1 phosphate at 961 cm⁻¹.

Fig 4. Representative Raman spectra of the ITD and PTD in the range from 800 to 1750 cm^{-1} . The spectra were normalized based on the peak intensity of CH_2 at $\sim 1450 \text{ cm}^{-1}$.

Fig 5.

Representative contact mode AFM images of fractured dentin surface before *in-situ* treatment with EDTA. Tubules are mainly parallel to the fractured surface. A and B, the topography and deflection images (in the scan area of 30 μm × 30 μm); C and D, the magnified images (in the scan area of 3.5 μm × 3.5 μm) of A and B, respectively

Fig 6.

Representative contact mode AFM images of the same fractured dentin surface represented in fig 6 after *in-situ* treatment with EDTA. Tubules are mainly parallel to the fractured surface. A and B, the topography and deflection images (in the scan area of 30 $\mu\text{m} \times 30 \mu\text{m}$); C and D, the magnified images (in the scan area of 3.5 $\mu\text{m} \times 3.5 \mu\text{m}$) of A and B, respectively.

Table 1Wavenumbers (in cm^{-1}) and peak assignments of micro-Raman spectra from the ITD and PTD*

ITD (cm^{-1})	PTD (cm^{-1})	Assignment
429 m	429 m	$\nu_2(\text{PO})^{[25, 42, 43]} (\text{E}_1/\text{A})^{[44]}$ in HAP
449 w	447 w sh	$\nu_2(\text{PO})^{[25, 43]} (\text{E}_2)^{[44]}$ in HAP
581 s	581 s	$\nu_4(\text{PO})^{[25, 42, 43]} (\text{E}_2)^{[44]}$ in HAP
590 s	590 s sh	$\nu_4(\text{PO})^{[25, 42, 43]} (\text{A})$ in HAP
609 m	608 m	$\nu_4(\text{PO})^{[25]}$ in HAP
854 w	853 w	$\delta(\text{CCH})^{[25]}$ aromatic (Pro, Tyr)
875 m	880 m	$\nu(\text{CC})^{[25]}$ aromatic (Hyp, Tyr)
961 vs	961 vs	$\nu_1(\text{PO}_4^{3-})^{[25, 43]} (\text{A}/\text{E}_2)^{[44]}$
1003 m	1003 m	$\nu(\text{CC})$ Phenyl ring ^[25]
1031 m	1031 m	Pyridine ring
1046 m	1046 m	$\nu_3(\text{PO})^{[25, 43]}$ out-of-phase; $\nu_3(\text{CO}_3^{2-}) (\text{E}^1)^{[44]}$
1071 ms	1071 ms	$\nu_1(\text{CO}_3)^{[25, 43, 45]}$ Type B Carbonate
1103 w		$\nu_1(\text{CO}_3)^{[45]}$ Type A Carbonate
1245 m	1241 m	$\delta(\text{NH})$ Amide III ^[25, 42, 45]
1268 m	1268 m	$\delta(\text{NH})$ Amide III ^{1, 2}
1450 m	1450 m	$\delta(\text{CH})$ deformation ^[25, 42, 46]
1670 m	1668 w	$\nu (\text{C}=\text{O})$ stretch/Amide I ^[25, 42, 46]
2882 m	2882 m	$\nu(\text{CH})$ asymmetric ^[25, 42, 45]
2939 s	2939 s	$\nu(\text{CH})$ symmetric ^[25, 45]
2982 m	2982 m	$\nu(\text{CH})$ symmetric ^[25, 42]
3064 vw	3064 vw	$\nu (\text{CH})$ aromatic
3325 w	3325 w	NH_2 and H_2O

* vw, very weak; w, weak; m, medium; s, strong; vs, very strong; sh, shoulder.

Table 2

The Raman intensity ratios of various peaks to the peak of CH₂ at ~1450 cm⁻¹ from the ITD and PTD

	ITD	PTD
	Mean ± SD	Mean ± SD
R _{1071/1450}	1.93 ± 0.17	5.40 ± 0.55
R _{961/1450}	12.85 ± 0.87	39.21 ± 3.01
R _{1670/1450}	0.91 ± 0.03	0.92 ± 0.05
R _{1245/1450}	0.72 ± 0.01	0.49 ± 0.06
R _{1268/1450}	0.59 ± 0.01	0.46 ± 0.01
R _{854/1450}	0.55 ± 0.04	0.31 ± 0.07
R _{875/1450}	0.33 ± 0.03	0.19 ± 0.04

Table 3

The Raman intensity ratios of various peaks in the region of 1010–1120 cm^{-1} to the peak of phosphate at $\sim 961 \text{ cm}^{-1}$ from the ITD and PTD

	ITD	PTD
$R_{1071/961}$	0.140 ± 0.003	0.132 ± 0.003
$R_{1031/961}$	0.053 ± 0.004	0.042 ± 0.005
$R_{1103/961}$	0.012 ± 0.010	0.007 ± 0.005