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A MICRO-RAMAN SPECTROSCOPIC STUDY OF HYDRAZINE-TREATED HUMAN DENTAL CALCULUS

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Abstract

Hydrazine has been used to remove organic components and to isolate the mineral(s) from human calculus. Micro-Raman measurements were performed on the mineral phase. After the hydrazine-treatment, not only a large reduction in fluorescence but also an increase in Raman signal was observed. The treatment was essential in minimizing thermally-induced chemical changes which could otherwise occur to the original calculus mineral due to the intense laser light. The Raman spectral features of the mineral were nearly all identical among the Raman spectra obtained at many randomly-selected sites by the micro-Raman microbe with a lateral resolution of approximately 1 μm, and were consistent with those of impure hydroxyapatite containing CO_3^{2-} and HPO_4^{2-}. The spectra contained typical hydroxyapatite bands including PO_4^{3-} bands of the ν_1, ν_2, ν_3 and ν_4 modes and one OH^- stretch band. Other minor bands due to the CO_3^{2-} ν_1 and ν_3 modes and bands possibly due to the HPO_4^{2-} ν_1, ν_2 and ν_4 modes were observable by the technique despite the hydrazine-treatment that could in principle remove the HPO_4 and CO_3 ions from the mineral. In comparison with pure synthetic hydroxyapatite, the intensity of the OH^- stretch band relative to that of the PO_4^{3-} ν_1 band was approximately 70% weaker, and the bandwidth of the phosphate ν_1 band was 200% broader, reflecting various crystal imperfections presumably present in the calculus mineral.

Key Words: Calculus, minerals, Raman spectra, hydrazine, hydroxyapatite, carbonate.

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Introduction

Calculus is mineralized plaque. Various mineral phases in dental calculus including hydroxyapatite (HAP), whitlockite (WH), octacalcium phosphate (OCP) and brushite (DCPD) have been reported [5, 9, 20, 21, 23, 24]. The most commonly used techniques in identifying these mineral phases have been the X-ray diffraction (XRD) and electron diffraction (ED) techniques. The former technique has been used for quantitative analysis, and compositional changes between newly formed and aged calculus have been observed [21, 24]. In addition, the constituent minerals identified in calculus varies among researchers.

Line-broadening of the XRD lines can occur if the crystal size is small and distortion is present in the crystal lattice. In fact, calculus mineral may contain a considerable amount of amorphous material [2, 11] and not be fully observable by XRD. This raises doubts about the XRD technique used for the mineral quantification in calculus though the XRD can detect selectively crystallized components which may be not observable with other techniques. Raman spectroscopy may provide an alternative means to gain more information on minerals which are crystalline and amorphous.

The electron diffraction technique has identified OCP [22] as well as HAP-like materials in calculus, but cannot be used for quantitative purposes. The infrared absorption technique (IR) could only confirm the spectra of carbonate hydroxyapatite in human dental calculus [15]. The previously published results on calculus using Raman spectroscopy showed little difference among different calculus formation periods though the Raman spectra were not comparable to those of any of the proposed mineral phases [27]. Since these Raman measurements employed initial laser irradiation with an increased laser power for fluorescence suppression, it was possible that the reported spectra could be partly from altered calculus due to laser heating. This problem can be circumvented by using hydrazine-treated calculus. The treatment is known to cause no significant interference with the original mineral, especially when amorphous

minerals are concerned [26].

In this study, the organic part of human dental calculus was removed by a hydrazine treatment, and the mineral part was examined by the micro-Raman technique. The detailed spectra of the hydrazine-treated calculus are presented and compared with those of synthetic minerals.

Materials and Methods

Sample preparation

Supragingival calculus formed on the lingual surfaces of the six lower front teeth (31, 32, 33, 41, 42, 43) was obtained from 18 adults (26-64 years; mean 43 years) by scaling at the end of a 6 month period by a dentist. During the experimental period, the patients used commercially-available fluoridated toothpastes without anticalculus agents. After calculus removal, the material collected was immediately washed on a filter under running tap water for 5 minutes to remove saliva and blood remnants. All the calculus obtained from the 18 patients was then mixed and stored dry.

Before Raman spectroscopic measurements, the organic part of calculus was removed by hydrazine treatment as follows. The mixed calculus was first ground by means of a mortar and pestle. 5 ml of an 85% aqueous solution of hydrazine hydrate $N_2H_4 \cdot H_2O$ (pH $\approx$ 12, Sigma Chemical Co., St. Louis, MO) was applied to 20 mg mixed calculus. The solution was agitated in a shaking machine at room temperature for 3 days, then fresh hydrazine was then added after discarding the old solution. This treatment was repeated, and the total duration of the treatment was thus 6 days. The hydrazine solution was discarded, and the calculus mineral rinsed three times with spectroscopic-grade acetone. Small droplets (10-30 μl) of the mineral-acetone suspension were placed on standard microscope slides and aluminum stubs for Raman spectroscopic and scanning electron microscopic (SEM) examinations, respectively.

Synthetic minerals

For reference purposes, Raman spectra of synthetic minerals including HAP (Hy-apatite, Eurocrystals, Landgraaf, The Netherlands), carbonated HAP prepared by the method described by Arends *et al.* [1], OCP (Procter & Gamble, Cincinnati, OH) and DCPD (Art. 2144, Merck, Darmstadt, Germany) were employed.

Raman measurements

An Ar^+ laser (515 nm, coherent; Innova 70, Palo Alto, CA, USA) was used as excitation source through a standard microscope (100X, NA = 0.95). Raman signals (180° back-scatter) were analysed by a spectrograph coupled with an image-intensified diode array detector (512 pixels, Peltier-cooled to -20°C; XX1630 Delft

Electronics, Delft, Netherlands; RL512S, EG&G, Sunnyvale, CA, USA). The overall spectral resolution was 6 cm^{-1} (full width at half maximum, FWHM). The focus spot size was approximately 1 μm, and the laser power approximately 20 mW. Microscope glass-slides can give a significantly high background when small calculus particles were examined; this problem was avoided by controlling the aperture in the optical system.

Raman measurements were carried out at randomly-selected sites on numerous randomly-selected calculus particles. Each measurement was allocated a serial number as shown in Figures 1, 3, 4, 5 and 6. (Note that the numbers indicate site, not sample.) In some cases, a higher laser power of 80 mW was used for 5 seconds to test the thermal effect on calculus. Background noises due to the dark current of the detector and spurious fluorescence and Raman emissions from the optical components were always subtracted from the raw Raman spectra. However, the background was not always completely subtractable on a whole spectrum. "Over"-subtraction can often cause artificial dips in some part of the spectrum. This was, however, avoided by applying the subtraction procedure to defined regions of the spectrum.

Scanning electron microscopy

A JEOL JSM-35 scanning electron microscope (SEM; JEOL, Tokyo, Japan) was operated at 25 kV, with a resolution of approximately 6 nm. Calculus samples were placed on 12 mm diameter aluminum stubs and gold plated using a standard sputtering equipment; the gold coating was 10-15 nm in thickness.

Results

Calculus prior to the hydrazine treatment exhibited a large fluorescence as shown in Figure 1a, masking spectral details. When a higher laser power (80 mW) was used for 5 seconds, the fluorescence was reduced significantly (Fig. 1c), and the spectra were identical to those reported previously [27]. The light absorption at the laser wavelength (515 nm) was high and caused partial carbonization of calculus material due to the consequential heating. This problem was not present in hydrazine-treated calculus. After hydrazine treatment, the background fluorescence was reduced significantly, and simultaneously Raman signals due the mineral components in calculus showed an increase in intensity (see Fig. 1b). The successful removal of organic materials was confirmed by the absence of bacterial structures within large calculus particles observed by SEM (Fig. 2a). The visible holes were approximately 1 μm in diameter and comparable to the size of plaque bacteria. No remnants of the original organic calculus matrix could be detected by SEM. In isolated cases, crystal-
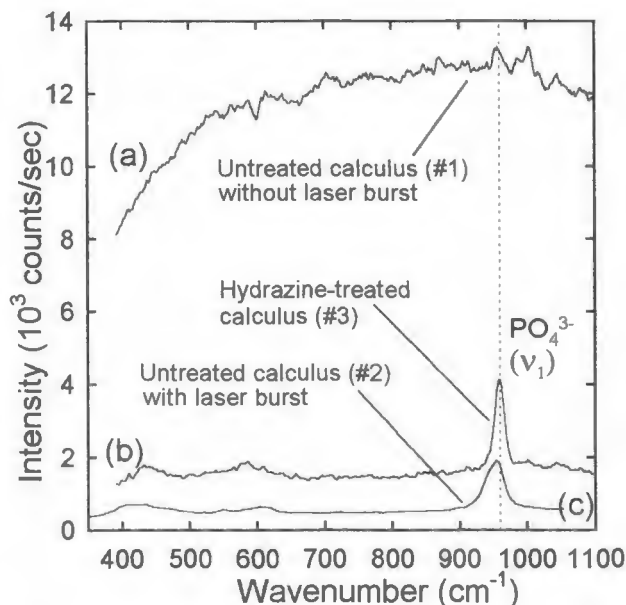
Figure 1. Fluorescence and Raman spectra of human calculus particles (#1-#3) of comparable size ($\sim 10 \ \mu$m). (a) Untreated calculus without initial laser irradiation. The spectrum shows a large fluorescence-background due to the large quantity of organic matrix present in normal calculus. (b) Hydrazine-treated calculus without initial laser irradiation. The level of fluorescence was reduced, and Raman signals due to the calculus minerals increased significantly. (c) Untreated calculus with initial laser irradiation at 80 mW for 5 seconds. This achieved the low background levels, but chemical alteration occurred. Conditions: laser power for the data acquisition: 20 mW, objective: 100X NA = 0.95.

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like structures, as shown in Figure 2b were noticeable. The type of mineral of these large crystals cannot be identified from the morphology alone (for calculus morphology see ref. [4, 13, 16]). Attempts were made to locate large crystals, initially by visual inspection through an optical microscope which is part of the micro-Raman spectroscopic system. The hydrazine-treated calculus was almost optically transparent for visible light and it was not possible to detect any crystals by visual inspection. Later, Raman measurements were performed on purpose at many different sites/particles as indicated in serial numbers (see Figures) to look for spectral differences.

Figure 3 shows a Raman spectrum of hydrazine-treated human dental calculus in the spectral range, 380-1100 cm^{-1}, together with spectra of synthetic minerals including DCPD, OCP, carbonated HAP and pure HAP. This calculus spectrum is an average of 13 individual spectra (shown in Fig. 4a) obtained after hydrazine treatment; these 13 spectra were taken in succession at 13
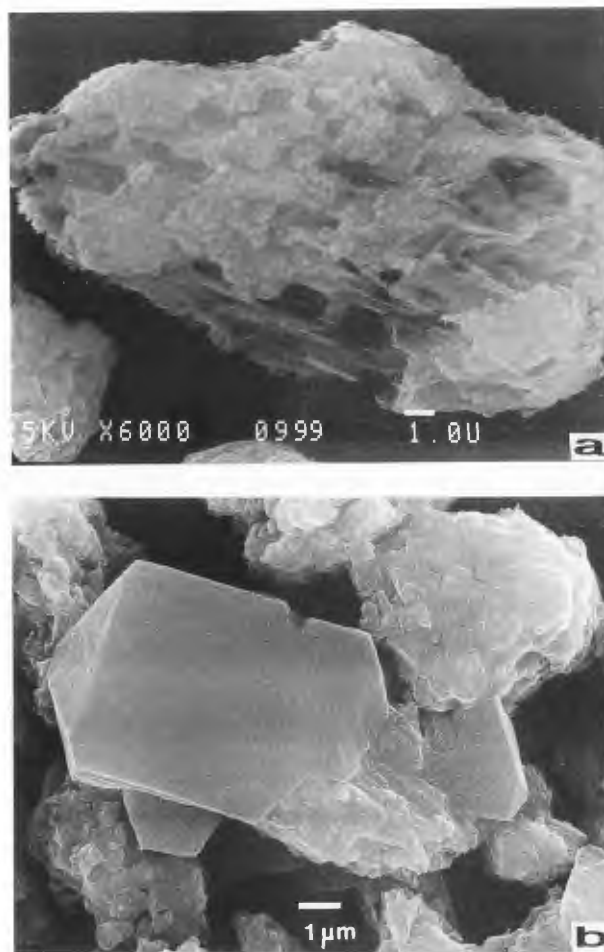
Figure 2. Scanning electron micrographs of hydrazine-treated human calculus. (a) A typical calculus particle showing holes which are indicative of removal of the organic matrix by hydrazine. (b) One of a few large crystals found in isolated cases in calculus clusters.

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different sites on 13 different calculus particles, and no preferential selection of data was made. The spectral regions were truncated to show the detailed spectral profiles. The minor broad background slopes or envelopes present in the original spectra (Fig. 3) were removed. (Note that this procedure provided more accurate and consistent results: the band positions were determined more precisely, and the band peak at $\sim$990 cm^{-1} in Fig. 3c, for example, was no longer seen after comprehensive background subtraction). The spectrum contained strong characteristic PO$_4^{3-}$ internal modes of ν_2/ν_4, ν_1 and ν_3 [10]. The CO$_3^{2-}$ ν_1 band at 1072 cm^{-1} and HPO$_4^{2-}$ band at 1003 cm^{-1} were observed in both spectra of hydrazine-treated calculus and synthetic carbonated HAP. Broad shoulder bands (400-410 / 520-560 cm^{-1}) were also noticeable where the HPO$_4^{2-}$ ν_2 and ν_4 bands are expected [6]. The CO$_3^{2-}$ ν_3 band at 1460 cm^{-1} was
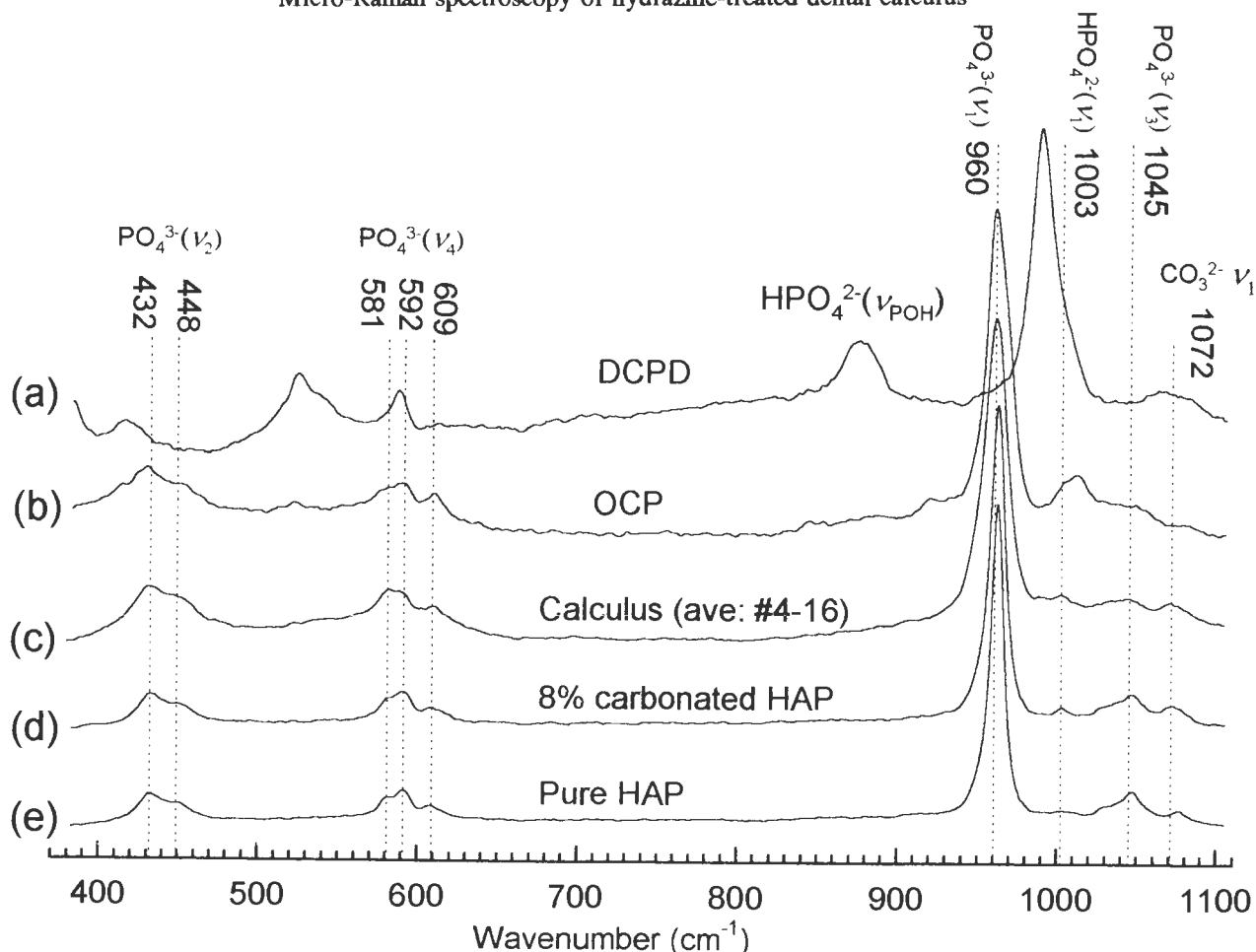
Figure 3. Raman spectra of hydrazine-treated human calculus and relevant synthetic minerals in the phosphate band region: (a) brushite (DCPD), (b) octacalcium phosphate (OCP), (c) hydrazine-treated calculus, (d) carbonated hydroxyapatite and (e) pure hydroxyapatite (HAP). The calculus spectrum (c) is an averaged spectrum of the individual spectra (shown in Fig. 4a) at 13 different randomly-selected sites/particles. The calculus spectrum resembles closely to that of synthetic carbonated HAP. Conditions: laser: 20 mW, 515 nm; objective: 100X, NA = 0.95; beam spot size: 1 μm, spectral resolution 6 cm^{-1}; total exposure time: approximately 500 seconds.

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weak but observable (see Fig. 5). The region below 380 cm^{-1}, where external modes of PO_4^{3-} and OH$^-$ are to be expected, was obscured by the strong background, and not explored.

The spectral features of hydrazine-treated calculus resemble closely those of synthetic carbonated HAP. The band positions of calculus mineral and synthetic minerals are summarized in Table 1. Raman spectra of calculus in the region of the OH$^-$ stretch band are shown in Figure 6. Two sets of spectra (Figs. 6a and 6b) were obtained from two separate experiments with slightly different spectrometer-setups. A slope (or envelope) present around the band in the original spectra was removed. The spectra of the synthetic minerals (Figs. 6d and 6e) are shown as reference for the band position and band width: the band intensities of these minerals have no relation to those of the calculus. However, by

taking two separate spectra (each covering the PO_4 or OH spectral region) at a given site under the same experimental conditions, the intensity of the OH band relative to that of the PO_4 bands can be compared. Figure 7 shows the (integrated) intensity of the OH$^-$ band versus that of the PO_4^{3-} ν_1 band for each site/particle. The slope of the straight line indicates the corresponding average intensity-ratio (or OH band intensity relative to the PO_4 band intensity). For hydrazine-treated calculus, this ratio was 70% lower than for pure HAP.

Discussion

The Raman spectra obtained from hydrazine-treated calculus contain typical PO_4^{3-} bands of the ν_1, ν_2, ν_3 and ν_4 modes, one hydroxyl stretch band, CO_3^{2-} bands and HPO_4^{2-} bands. These spectral features were very con-
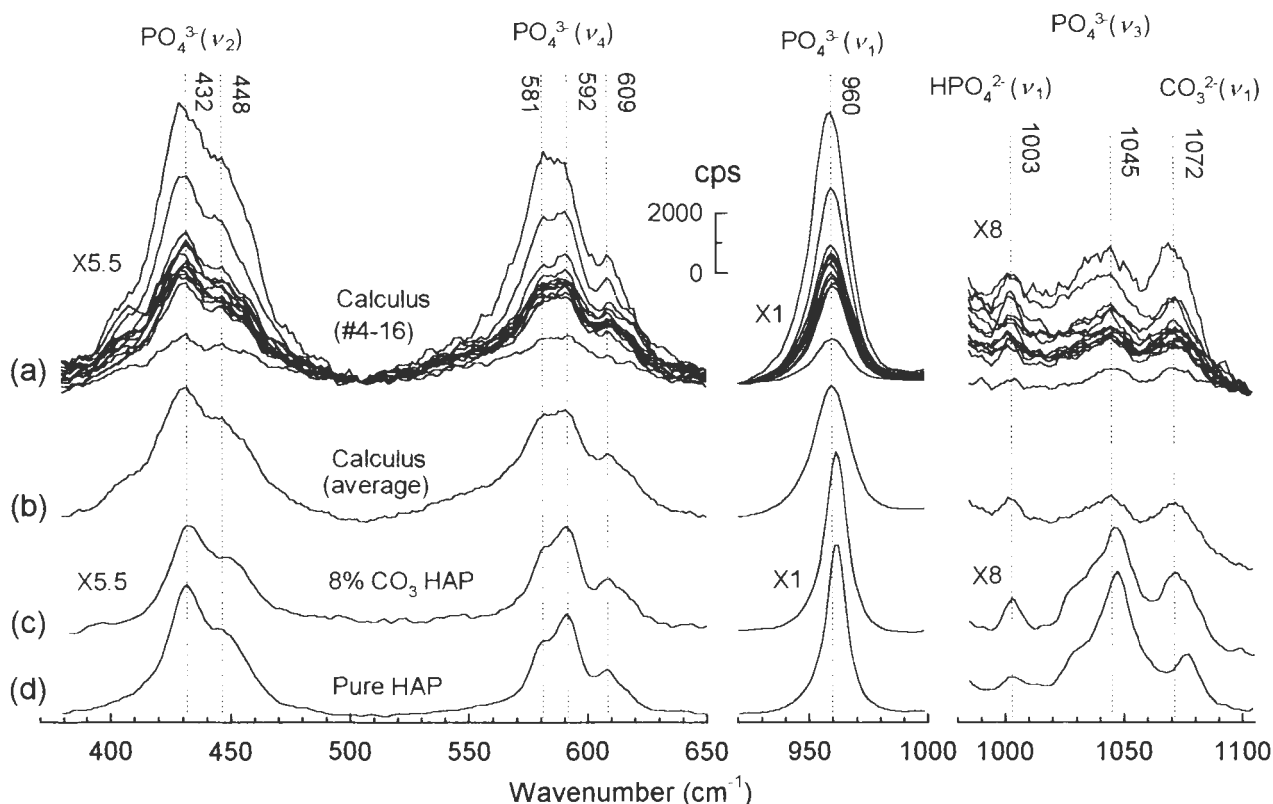
Figure 4. Spectra of hydrazine-treated human calculus and synthetic minerals showing the PO_4^{3-}, HPO_4^{2-} and CO_3^{2-} bands: (a) hydrazine-treated human calculus. Each spectrum was taken from a randomly-selected site/particle. (b) Averaged spectrum of hydrazine-treated human calculus (#4-16). (c) Synthetic carbonated HAP. Carbonate concentration: 8 wt%. (d) Pure HAP. Spurious background noises as well as slopes and envelopes present in the original spectra (Fig. 3c) have been subtracted carefully. Conditions: laser: 20 mW, 515 nm; objective: 100X, NA = 0.95; beam spot size: 1 μm, spectral resolution 6 cm^{-1}; total exposure time: approximately 500 seconds.
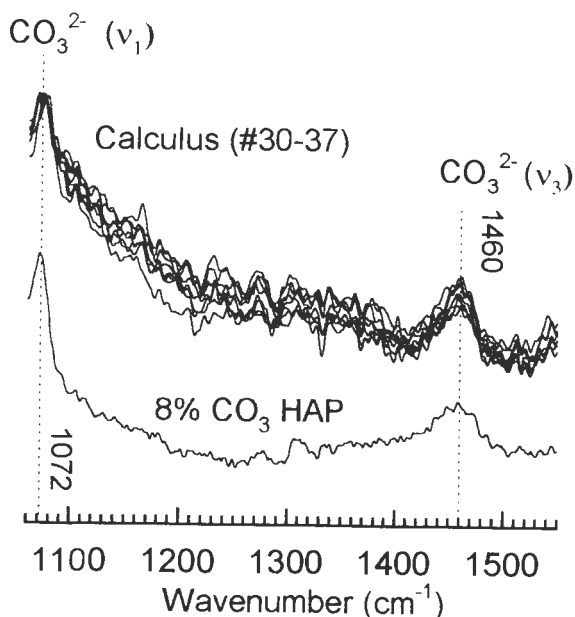
Figure 5. Raman spectra of hydrazine-treated human calculus and synthetic carbonated HAP in the spectral region of the CO_3^{2-} ν_1 and ν_3 bands.

sistent with those of synthetic carbonated HAP [17], which also contains HPO_4^{2-} [6] as impurity. There were, however, minor differences in bandwidths and band positions of the PO_4^{3-} ν_1 and OH^- stretch band. The strongest PO_4^{3-} ν_1 band was consistently at a slightly lower wavenumber by 2 cm^{-1}. The bandwidth of this band was approximately 8 cm^{-1} (200%) broader than the corresponding band of pure synthetic HAP. The position and bandwidth of the ν_1 band could be influenced by the presence of crystal impurities. Previously, the position has been shown to be red-shifted by 2-4 cm^{-1} in synthetic carbonated HAP and human enamel [19]; the bandwidth of the PO_4^{3-} ν_1 band has been found to increase approximately linearly with the carbonate concentration up to a very high carbonate concentration [17], though linearity occurred only below 8% carbonate concentration in other experiment [3]. It is possible that not only carbonate ions but also other impurities contribute to the disorder in the crystals. There are discrepancies among the reported results concerning the bandwidth of PO_4^{3-} band and intensity of CO_3^{2-} band, and are most likely due to completely different preparation methods

Table 1. Spectral positions of the observed Raman bands of human calculus and synthetic minerals.

	(Band positions in cm^{-1})			
Human calculus	Carbonated HAP	Pure HAP	OCP	Mode of vibration
400-410				$HPO_4^{2-}\ \nu_2$
432	432	432	428	$PO_4^{3-}\ \nu_2$
448sh	449sh	448sh	448sh	
520-560			525	$HPO_4^{2-}\ \nu_4$
581	581	581	585	$PO_4^{3-}\ \nu_4$
592	591	592	594	
609	609	609	615	
	617sh		617sh	
960	962	962	959	$PO_4^{3-}\ \nu_1$
			965sh	
1003	1003		1003sh	$HPO_4^{2-}\ \nu_1$
			1011	
	1028sh	1034sh		$PO_4^{3-}\ \nu_3$
1045br	1048	1048	1048	
1072	1072			$CO_3^{2-}\ \nu_1$
	1077	1077	1075	$PO_4^{3-}\ \nu_3$
1460	1460			$CO_3^{2-}\ \nu_3$
3571	3572	3572		OH$^-$ stretch

The band positions were determined after removing slopes (or envelopes) present around the bands in the original calculus spectra (Fig. 3c), and slight differences in the band positions may be seen (cf. Figs. 3c and 4b). For detailed assignments for the synthetic minerals, see references [6, 17]. sh: shoulder; br: broad band.

Table 2. Summary of the identified calculus minerals.
(HAP: hydroxyapatite; OCP: octacalcium phosphate; DCPD: brushite; WH: whitlockite)

Year	Technique	Minerals identified	Reference
1954	X-ray diffraction	HAP, WH, DCPD	(11)
1960	X-ray diffraction	HAP, WH	(4)
1964	X-ray diffraction	HAP, WH, OCP, DCPD	(21)
1965	electron diffraction	HAP-like	(13)
1966	X-ray diffraction	HAP, WH, OCP, DCPD	(23)
1967	X-ray diffraction	HAP, WH, OCP, DCPD	(8)
1968	electron diffraction	HAP, OCP	(22)
1973	X-ray diffraction	HAP, DCPD	(20)
1974	infrared absorption	HAP (CO_3^{2-})	(14)
1994	micro-Raman	HAP (CO_3^{2-}, HPO_4^{2-})	Present work
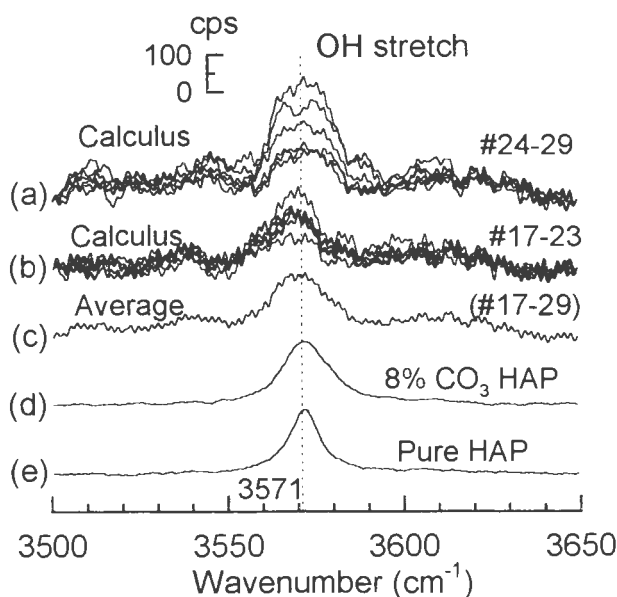
Figure 6. Raman spectra of hydrazine-treated human calculus and synthetic carbonated HAP in the OH stretch region. (**a**) Hydrazine-tread calculus (one set of measurements: sites #24-29). (**b**) Hydrazine-treated calculus (another set of measurements done earlier: sites #17-23). (**c**) Hydrazine-treated calculus 13-average (#17-29). (**d**) Synthetic 8 wt% carbonated HAP. (**e**) Synthetic pure HAP. The slight difference between (a) and (b) is due to changes in the instrumental setup. Slope and envelopes in the original spectra have been removed. Conditions: laser: 20 mW, 515 nm; objective: 100X, NA = 0.95; beam spot size: 1 μm, spectral resolution 6 cm^{-1}; total exposure time: approximately 500 seconds.

Figure 7. The Raman intensity of the OH stretch band is plotted against that of the PO$_4^{3-}$ ν_1 band for each measurement on hydrazine-treated human calculus, synthetic pure HAP and carbonated HAP. The gradient of each straight line through the origin represents the corresponding average ratio of the OH band intensity and PO$_4^{3-}$ ν_1 band intensity. Conditions: laser: 20 mW, 515 nm; objective: 100X, NA = 0.95.

used, leading to carbonate ions at different lattice sites. The bandwidth of the PO$_4^{3-}$ ν_1 band can not be used as a basis to estimate the carbonate concentration in the HAP crystal. The intensity of the carbonate ν_1 band is, however, consistent with 2-8 wt% carbonate by judging from the results by Nishino *et al.* [19] and Nelson *et al.* [17]. Substitution of CO$_3^{2-}$ into OH positions has been seen previously as cause of a drop in OH band intensity [19]. The low intensity of the OH stretch band of calculus indicates a large deficiency of OH ions possibly caused by vacancies, ion substitution and Ca-deficiency. The broader bandwidth of the PO$_4^{3-}$ ν_1 band can also account for these effects. If the OH band intensity is approximately proportional to the OH concentration in the mineral crystal, the amount of OH would be equivalent to approximately 30% of pure stoichiometric HAP.

A potential concern is the alteration of the original mineral phases (present in untreated calculus) by the laser irradiation and by the hydrazine treatment. The previous Raman studies reported no great differences in mineral variations among sites and age of human calculus [27], although some chemical changes might have been caused by heat due to the presence of light absorbing organic components during the laser irradiation used on untreated calculus. The reported spectra differed from those presented in this paper. Some thermal alteration to the original mineral in calculus in the previous results has to be suspected. Raman spectroscopy relies on the balance between suppression of fluorescence background and increase in signal intensity. It is always advantageous when higher laser power is used since the fluorescence can be bleached and the Raman signals can be increased at the same time.

The micro-Raman setup with back-scattering geometry used in this investigation is suitable for highly absorbing materials [28], but the power density of the focussed laser beam can be high when an objective lens with a short focal length is used. High power lasers, such as CO$_2$ and YAG (yttrium-aluminum-garnet) lasers, have been used to modify enamel surfaces [8, 25].

Especially with a CO_2 laser, at the laser wavelength, HAP has a very high absorption and the heat can be generated in a thin volume resulting in melting and glassy formation on the surface. It has been shown that the induced changes caused the formation of tetracalcium phosphate monoxide $Ca_4(PO_4)_2O$ [18]. The peak power density delivered by the CO_2 laser was in the range of 10-100 GW/cm^2. In the case of the micro-Raman technique used in our study, the diameter of the focussed laser beam on the sample was approximately 1 μm with laser power 20 mW, which gave the power density of 2.6 MW/cm^2. This is 3 orders of magnitude lower than that of the pulsed lasers mentioned above, and absorption of light by HAP at 515 nm is negligibly small compared with far-infrared at 10 μm. Biological materials not only absorb the laser light but also act as heat-insulator around the calculus minerals. This prevents the heat from escaping, due to its poor thermal conductivity especially when dry, and this may lead to local heating. The level of heat trapped in untreated calculus may reach a level high enough to alter the original calculus mineral even with a low-power continuous-wave laser. After hydrazine treatment, such a thermal problem is, however, expected to be negligible, since the light-absorbing components (which could also act as thermal insulator) were removed and heat-conducting mineral was concentrated. The intensity and position of the OH stretch band can be affected by high temperatures, but there was no observed change before and after increasing laser power up to 80 mW. Spectral features of the phosphate bands also remained unchanged by this power increase. The chemical changes caused by laser-heating are therefore unlikely, and the mineral spectra observed with this technique are indeed from the mineral of the hydrazine-treated calculus, but not of new thermally-induced mineral phase.

Another potential concern is the possible changes in the mineral due to hydrazine which has been used successfully to study minerals in bone and teeth [26, 29]. It has been reported that hydrazine can cause chemical alterations of minerals. The acidic groups present in minerals such as HPO_4^{2-} and CO_3^{2-} can be lost in the strong alkaline conditions of the hydrazine treatment. Such losses can occur on the crystal surfaces, but would be small if these ions are in the bulk of the crystals. The induced loss of HPO_4 for example is reported to be 30-35% [29]. In our study, the relatively long hydrazine-treatment of 6 days was necessary for the substantial removal of organic components from the original calculus. The effect of the hydrazine treatment on calculus minerals can be greater than suggested. This means that the observed CO_3^{2-} and HPO_4^{2-} bands might have been under-detected, and the concentration of other mineral phases containing HPO_4^{2-} may have become too low for the Raman technique.

Table 2 summarizes the mineral phases identified previously by various techniques in human dental calculus. The previous reports on mineral phases in calculus using electron diffraction technique suggested that mineral-changes could occur after a prolonged exposure to the electron bombardment. Apatite-like diffraction patterns have been reported [14, 23]. Some of these are considered to be an artefact caused by high energy electrons, though the exact nature of the changes are unclear. By cooling calculus samples with liquid nitrogen, an additional mineral phase, OCP, has been reported [22]. However, there has been no quantitative report using electron diffraction technique.

To date, the quantitative identification of mineral phases of calculus has been based on older XRD data [5, 9, 20, 21, 24]. The XRD technique relies on analysis of interference patterns of the de/reflected waves from an extremely large ensemble of regularly spaced diffracting centers cooperating coherently. The coherent cooperation by the extremely large lattice ensemble can be interrupted by changes in lattice parameters and crystal size (if the crystal size is smaller than the coherent length of the X-ray spontaneous emission line used). It has been shown that the diffraction pattern of HAP in enamel was quite sensitive to the crystal size [7]. Furthermore, Jenkins [11] and Arends et al. [2] indicated a sizeable amount of amorphous HAP-like materials present in calculus. The XRD technique may, therefore, ignore the major part of calculus material, especially if the crystals are not large and ordered enough to provide diffraction patterns with sufficient contrast, as indicated by the presence of CO_3 and HPO_4 ions and the broad bandwidth of the PO_4^{3-} ν_1 band of calculus apatite found in this study. The authors consider the XRD technique, in this case, not quantitative. In contrast, Raman spectroscopy as well as infrared absorption spectroscopy rely on the local crystal symmetry, and are therefore less sensitive to the lattice disorder or crystal size. If different mineral phases exist in comparable concentrations, the Raman technique may provide better overall information concerning the constituency of mineral phases.

Impure hydroxyapatite detected by the Raman technique after hydrazine treatment in this study is confirmatory to the results reported previously using the infrared technique on human calculus [15]. The full extent of mineral alteration to calculus minerals by the hydrazine treatment and the end-phase of such alteration are not known. In addition, the calculus used in this study contains large amounts of F due to the fluoridated toothpaste used by the patients. Considerable amounts of F may lead to the formation of calculus containing predominantly the HAP phase because intermediate phases such as DCPD and OCP can transform into HAP.

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Discussion with Reviewers

T. Kodaka and M. Suzuki: Human supragingival calculus usually contains OCP as well as biological apatite (AP). Is the absence of the peak at 1011 cm^{-1} due to OCP enough to conclude that OCP was not detected in the calculus samples #4-166 (Fig. 3)? How are the

other samples (#1-3, #17-29)?

Authors: It is likely that the concentration of OCP relative to that of the major mineral (HAP) in **hydrazine-treated** calculus was not high enough for the technique to distinguish the OCP peak at 1011 cm^{-1} from the adjacent PO$_4$ ν_3 and HPO$_4$ ν_1 bands. Hydrazine and fluoridated toothpaste might, however, cause appreciable effects on OCP. Hydrazine has been known to remove acidic groups of minerals, and OCP, DCPD and whitlockite may have been affected by the treatment. The extent of hydrazine-extraction may depend on, for example, the crystal size of calculus-mineral, and is difficult to evaluate. In addition, toothpaste used by the patients contained fluoride, and the calculus is expected to contain large amounts of F which may also transform DCPD and OCP into HAP. To clarify this problem, a further Raman spectroscopic study on calculus is currently in progress using a near-infrared Fourier transform Raman spectrometer, which enables us to observe Raman spectra without hydrazine treatment. (Each number corresponds to a randomly-selected *site* on randomly-selected particles of ground mixed calculus, thus not a sample.)

T. Kodaka and M. Suzuki: Supragingival calculus collected at 6 months after cleaning and polishing, probably contains DCPD besides OCP and AP [24]. The large crystal shape shown in Figure 2b is similar to that of DCPD [13, 16]. Moreover, the Raman spectrum in Figure 3c detected a weak peak around 989 cm^{-1}: the wavenumber of which coincides with that of the strongest peak of DCPD in Figure 3a. These facts indicate the presence of DCPD in the calculus. Please comment.

Authors: Large crystals were found **in isolated cases** by SEM. It was, however, not possible to locate any crystal formation through an optical microscope as part of the micro-Raman spectroscopic system, since hydrazine-treated calculus was almost optically-transparent for the visible light. Therefore we made an attempt, instead, to detect spectral variations or different band-peaks by measuring at **many sites/particles** with the micro-Raman technique as described in the paper. In general, identifying various calcium phosphate phases by the morphology alone is not appropriate unless there is an independent evidence. In our case, the band at $\sim$990 cm^{-1} was found to be spurious background possibly caused by glass or anti-reflection coating on objective lens. The phase of the crystal is, therefore, unknown.

T. Kodaka: Fluoride causes DCPD to transform into HAP through OCP (DCPD → OCP → HAP) [4, 20, 16]. Why did you use fluoride-containing dentifrice during the period of calculus formation? What is the effect of toothbrushing with fluoride dentifrice for 6 months?

Authors: All commercially available toothpastes in Europe contain fluoride. It is nearly impossible for ethical and legal reasons to do a study without fluoride in the dentifrice. The transformation from DCPD → OCP → HAP is known to be strongly controlled by the presence of fluoride. Fluoride shifts the presence of DCPD to apatite. The amount of fluoride in calculus from numerous sources is presumably quite high. Tentatively, the high F levels in calculus may cause a fast shift of acidic phases DCPD or OCP to HAP. Because micro-Raman experiments on calculus with a low F content are unknown, one can speculate that the observation in the paper that the main mineral phase in calculus is HAP containing CO$_3$ and HPO$_4$ is strongly influenced by the presence of F from dentifrice.

T. Kodaka: Biological apatites are probably Ca-deficient or defective HAP as well as carbonated HAP [4, 16]. Can micro-Raman measurement detect Ca-deficient HAP?

Authors: The question is difficult to answer since there have been no Raman-spectral data concerning Ca-deficient HAP. However, the Raman technique can detect signals due to phosphate vibrations of both defective and non-defective HAP without significant intensity changes. This property has been used to study substitution mechanisms in HAP [3, 17]. Carbonate substitution is known to cause a band-broadening of the PO$_4^{3-}$ ν_1 band of HAP, and is accountable for the observed band-broadening. The carbonate-substitution (as well as fluoride-substitution) in apatite might be accountable for the observed low relative-intensity of the OH stretch band (or OH/PO$_4$ intensity ratio) in calculus.

T. Kodaka: Sodium hypochlorite (NaOCl) also dissolves organic debris of dental calculus [13]. Are the effects of NaOCl and hydrazine similar?

Authors: Hypochlorite and hydrazine are both alkaline chemicals, and the effects on organic and mineral components in calculus are expected to be similar.