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Fluorescence spectroscopy of dental calculus

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Background: Correct diagnosis of the presence and extent of subgingival calculus is important for periodontal treatment planning and reassessment after periodontal therapy. Traditional tactile methods often lack sensitivity. The present investigation shall contribute to understanding the fundamental fluorescence properties that may be useful for optical detection of both supra- and subgingival calculus.

Objectives: The aim of this study was to investigate emission spectra from supra- and subgingival calculus under a wide range of excitation wavelengths.

Methods and Results: Extracted human molars with either supragingival or subgingival calculus deposits on the root surface were selected ($n = 3$ each). Emission spectra were recorded from the calculus of each tooth and corresponding areas of clean root surfaces using a fluorescence spectrophotometer at excitation wavelengths from 360 nm up to 580 nm in steps of 20 nm. The spectra were corrected for the wavelength dependent instrument sensitivity and normalized to peak intensity (the highest peak was set at 1.0). Emission spectra of calculus exhibited distinct fluorescence bands between 570 and 730 nm not present in clean root surfaces. This fluorescence emission was strongest for excitation wavelengths from 400 to 420 nm. No differences were observed between supra- and subgingival calculus.

Conclusions: Human dental calculus can clearly be differentiated from clean root surfaces by emission spectrophotometry. The characteristic fluorescence emission of supra- and subgingival calculus may be due to a variety of porphyrin derivatives and may provide the basis for future diagnostic procedures.

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Subgingival debridement including disruption of microbial biofilm and removal of subgingival calculus is an effective measure for treatment of chronic periodontal disease (1). Objective determination of presence and extent of subgingival calculus is important for both diagnostic decision making before treatment and checking the results thereafter. With traditional assessment methods, probing and radiography, subgingival calculus detection is often difficult and not reproducible (2, 3). To overcome the problem, means have been suggested that rely on miniature fiberoptic endoscopes (4) or electronic measurement devices (5). Promising methods suggested for detection of subgingival calculus are based on differences of fluorescence properties of calculus and dental hard tissue (6, 7). Although fluorescence of dental calculus has been known for some time (8), systematic investigations into fluorescence properties of supra- and subgingival calculus are scarce. In order to get deeper insight into the complete fluorescence emission behavior of dental calculus and adjacent unaffected tooth structure, systematic investigations using multiple excitation wavelengths are necessary. These data may serve as a basis for further development of optical detection technologies for periodontology.

The aim of this investigation was to compare fluorescence properties of both supra- and subgingival calculus with clean root surfaces using a wide range of excitation wavelengths in the ultra violet (UV)-visible range.

Materials and methods

Specimen selection

Twelve teeth extracted for periodontal reasons with supragingival ($n = 3$) and subgingival calculus ($n = 3$) and with exposed root dentine ($n = 6$) were selected. The surfaces of the teeth were gently cleaned by hand with a toothbrush under running tap water. Hereby the surfaces of the exposed roots and calculus deposits were left intact. All teeth were stored in 0.1% thymol solution at 4°C in the dark until the measurements were carried out.

Spectrophotometry

Emission spectra were recorded using a modified spectrophotometer (LS 50B, Perkin Elmer Ltd, Beaconsfield, UK). The spectrophotometric set-up (Fig. 1) comprised a pulsed Xenon discharge lamp for excitation and a grating with 1440 g/mm for wavelength selection. The excitation side of the spectrophotometer was also fitted with a beam-splitter and reference photomultiplier tube (PMT) to record excitation intensity for correction purposes. The emission side of the spectrophotometer comprised a grating (1200 g/mm) for wavelength selection and a red sensitive PMT (R955, Hamamatsu Photonics K.K., Shimokanzo, Japan) for emission detection. Emission and excitation slit widths, PMT voltage and scan parameters were controlled by a computer program (FL WinLab, Perkin Elmer Ltd) but determined by the operator. The whole teeth were fitted into a custom-made sample chamber ($40 \times 40 \times 40$ mm^3). The chamber was filled with water and had quartz-glass windows for improved UV-violet transmittance in the excitation and emission path. It housed a specimen holder which could be aligned in three axes and rotated so that the excitation beam hit the tooth surface at an angle of 30° (60° to the surface normal). The emission path was 90° to the excitation path and 60° to the tooth surface (30° to the surface normal) to suppress surface reflection. The teeth were mounted within the sample chamber so that either the calculus area or the clean root surface free from calculus was exposed to the excitation beam. Continuous fluorescence emission scans up to 800 nm were performed at a scan speed of 400 nm/min for 12 excitation wavelengths from 360 up to 580 nm at 20 nm intervals. Hereby, the emission signal was recorded at intervals of 0.5 nm. In between two light pulses from the Xenon discharge lamp a dark signal was recorded and subtracted from the emission signal (background correction). Both, excitation and emission slit widths were set at 3–16 nm to achieve high signal levels below PMT saturation threshold. To suppress stray light and higher order artefacts, individual long pass filters (Schott Glas, Mainz, Germany) were inserted in the emission path with cut-on wavelengths 30 nm higher than the excitation wavelength used. To correct for fluctuations in lamp intensity the signal from the emission PMT was ratioed by the intensity of the excitation signal. Excitation scans from 300 to 580 nm were made with the same experimental set-up from every specimen for those emission wavelengths

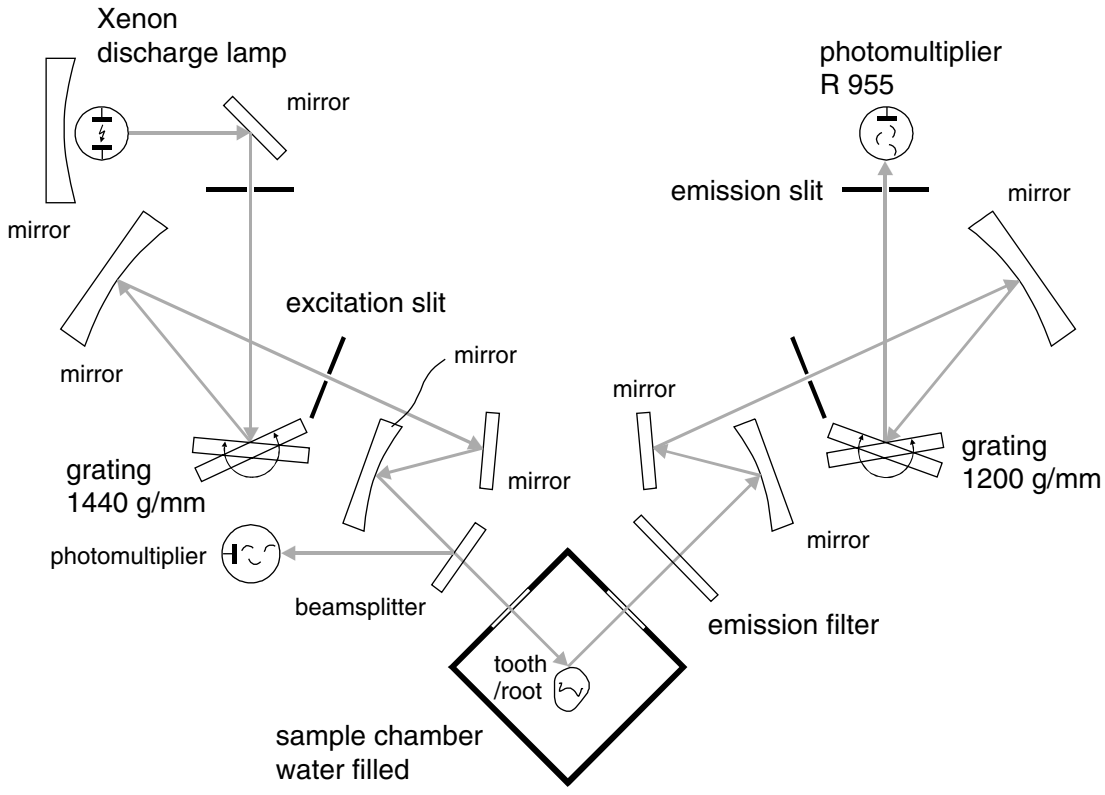

Fig. 1. Schematic illustration of the spectrophotometric instrumentation (not to scale). The path of light is indicated by arrows.

that showed peak intensity in emission scans of calculus. The signal from the emission PMT was ratioed by the intensity measured by the excitation PMT.

Post measurement calculations

All collected spectra were corrected for emission sensitivity of the experimental setup by an intensity curve derived from a calibrated QTH lamp (LS-1-CAL, Ocean Optics Inc., Dunedin, FL, USA) that was traceable to an intensity standard provided by the National Institute of Standard and Technology (NIST).

Emission spectra were smoothed using a moving average filter with 11 point filter width (corresponding to 5 nm wavelength width). Emission and excitation spectra were normalized to peak intensity (peak intensity set at 1.0). Excitation–emission matrices were calculated for the averaged spectra from clean root surfaces and the spectra from supragingival and subgingival calculus.

Results

Clean root surfaces exhibited an emission peak close to the excitation wavelength and continuously decreasing intensity towards higher wavelengths. For a distinct excitation wavelength, only negligible differences were observed between different teeth. The emission peak of clean root surfaces was dependent on the excitation wavelength (Fig. 2A). The emission intensity decreased with increasing excitation wavelengths, which cannot be seen in normalized Excitation–emission matrices.

Emission spectra from supra- and subgingival calculus were more irregular when different teeth were compared than spectra from clean roots, but no characteristic features could be identified that allowed distinction between supra- and subgingival calculus. However, emission spectra from calculus were clearly different from clean root spectra. All emission spectra from supragingival calculus (Fig. 2B) and subgingival calculus (Fig. 2C) had high intensity peaks between 570 and

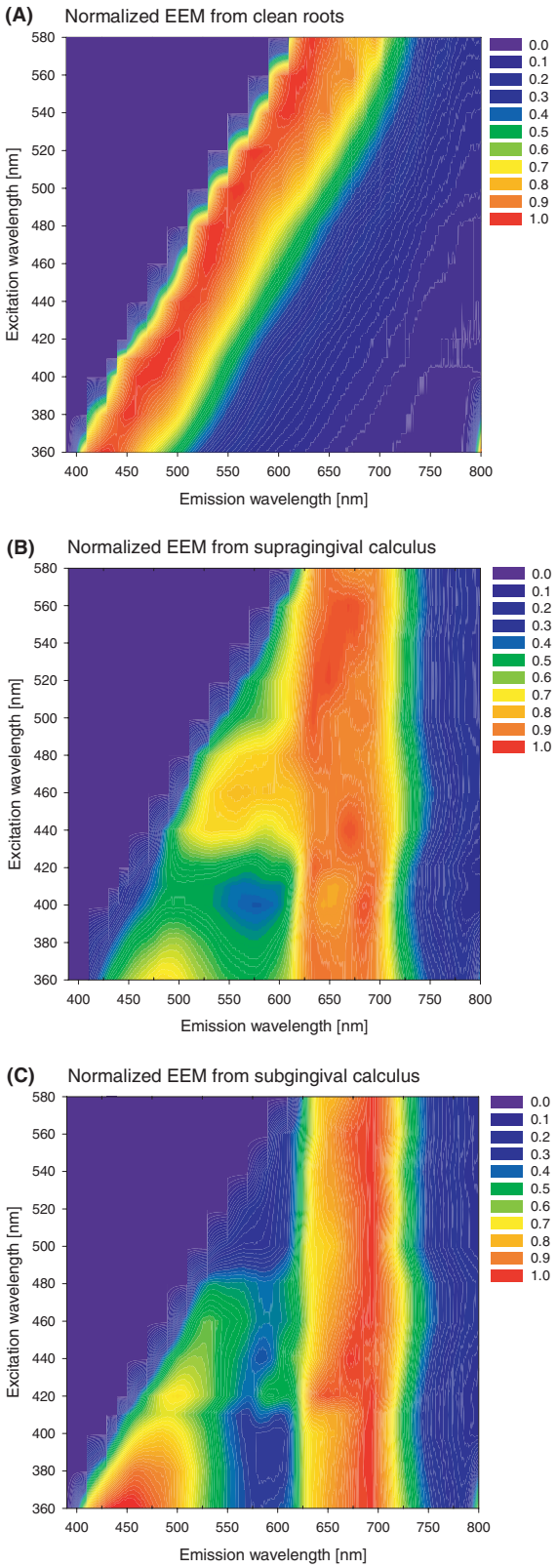

Fig. 2. Excitation-Emission Matrices (EEMs) normalized to peak intensity. Emission intensity is color coded and expressed as a function of excitation and emission wavelength. (A) EEM averaged from six clean root surfaces. (B) EEM averaged from three supragingival calculus specimens. (C) EEM averaged from three subgingival calculus specimens.

740 nm that were independent from the excitation wavelength. Although the position of these peaks was independent from excitation, the intensity was dependent on the excitation wavelength, which cannot be seen in normalized spectra. Emission intensity between 570 and 740 nm was highest for excitation wavelengths around 400–420 nm. Single emission spectra at 420 nm excitation taken from supragingival calculus (Fig. 3A) and subgingival calculus (Fig. 3B) showed high intensity peaks between 635 and 695 nm and a peak at 595 nm not present in clean root surfaces. The peak present in clean root emission spectra, e.g. around 495 nm for 420 nm excitation, however, was found in all spectra from calculus as well. The excitation spectra taken from supragingival calculus at 633 nm showed its highest intensity around 400 nm whereby two single peaks at 398 and 405 nm could be resolved (Fig. 4). Smaller peaks were present at 507 and 538 nm.

Discussion

Fluorescence properties of both supra- and subgingival calculus may provide a means for accurately assessing presence of supra- and subgingival calculus in regions not directly accessible to the eye or difficult to reach by periodontal probing. Although fluorescence of calculus has been known for some time (8), more systematic investigations were undertaken only recently (9–11). Study methods in these, like in most studies, involved fluorescence measurements with dissolved calculus samples. In the present study fluorescence spectra were recorded from solid calculus directly with a view to possible clinical application.

Excitation of all clean root surfaces with the same wavelength resulted in emission spectra with only negligible differences. Similar spectra with a broad peak close to the excitation wavelength and a continuous decrease towards higher wavelengths are known from previous studies on enamel for a few excitation wavelengths (12–16). It became evident from all emission spectra that the emission peak of the clean root areas moved towards longer wavelengths with increasing excitation wavelength. A similar emission peak at the same position was found for all emission spectra taken from calculus as well, i.e. around 495 nm for 420 nm excitation (Figs 3A and B). Emission peaks that move towards longer wavelengths with increasing excitation wavelengths are known from vibrational effects, i.e. Raman scattering rather than fluorescence. Emission peaks due to fluorescence typically remain at their wavelength position but change intensity with changing excitation wavelengths. Hence, it can be assumed that the broad intense emission peaks of clean roots (Fig. 2) as well as the corresponding emission peaks of dental calculus are not due to fluorescence. Only presence of multiple fluorophores that cover a wide range of excitation and emission maxima and simultaneous quenching and reabsorption effects may explain 'moving peaks' caused by fluorescence. Further research may be required to find out the cause for these emission peaks. However, the peaks in the red spectral region between 570 and 740 nm present in calculus preserve their wavelength position independently of the excitation wavelength

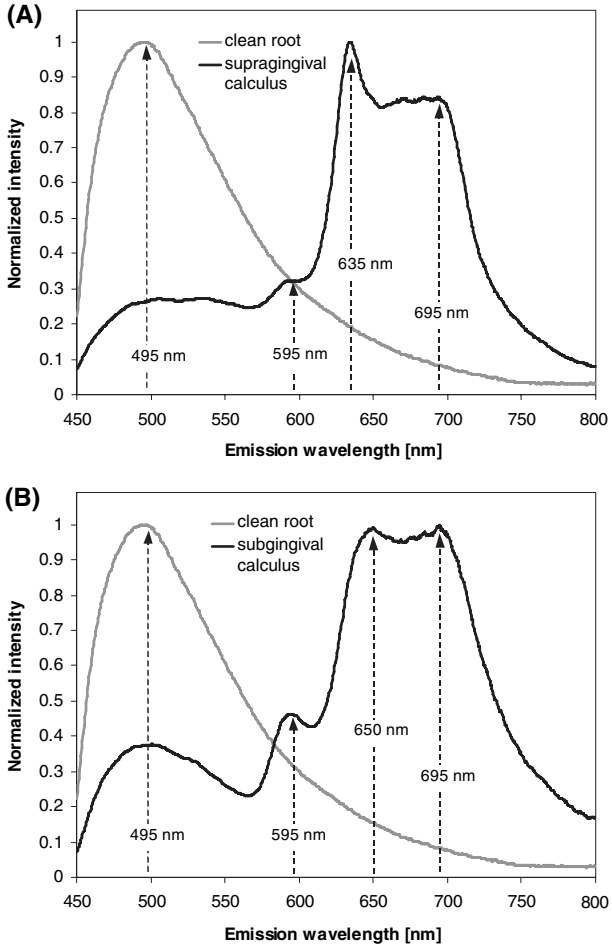

Fig. 3. Emission spectra averaged from clean root surfaces, and from single supra- and subgingival calculus areas for 420 nm excitation normalized to peak intensity. (A) Clean root (gray line) and supragingival calculus (black line). Supragingival calculus showed emission peaks at 595 and 635 nm and a shoulder with highest emission intensity at 695 nm not present in clean root surfaces. Both clean root and supragingival calculus had a peak around 495 nm. (B) Clean root (gray line) and subgingival calculus (black line). Subgingival calculus showed emission peaks at 595, 650 and 695 nm and peaks that could not be resolved between 650 and 695 nm, all of them not present in clean root surfaces. Both, clean root and supragingival calculus had a peak around 495 nm.

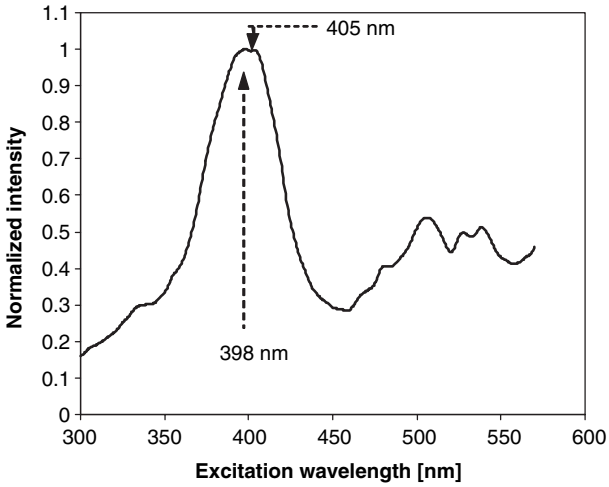

Fig. 4. Excitation spectra from the same supragingival calculus specimen as Fig. 3A recorded at 633 nm emission. Highest excitation was induced at 398 and 405 nm corresponding to the Soret bands of at least two porphyrin derivatives.

(Figs 2A and B) which is indicative of fluorescence. Peaks in this range were present in all calculus samples. Highest emission intensity in the 570–740 nm range was obtained with excitation between 400 and 420 nm that is in accordance with the Soret band of porphyrins around 405 nm. Specific fluorescence emission peaks could be resolved at 595, 635, 650 and 690 nm, but the broad shoulder between 650 and 695 may hide further distinct fluorescence peaks that could not be resolved with the applied technique. The emission peak of supragingival calculus at 635 nm (Fig. 3A) was highly excited by 398 and 405 nm and to a lesser degree by 507 and 538 nm. This may be indicative of at least two porphyrin derivatives with Soret bands around 405 nm and less intense Q bands at higher wavelengths. Thus, it can be assumed that a mixture of different fluorescent chromophores is present in human calculus as has already been suggested (9). It was beyond the scope of this study to extract specific molecules responsible for the characteristic fluorescence peaks in the red spectral region. However, emission peaks at 595 nm and 635 nm have already been observed in carious enamel and were suspected to be due to the presence of Zn-protoporphyrin and protoporphyrin IX (17).

It is known that porphyrin fluorescence found in dental calculus as well as in carious dental hard tissue is closely linked to high bacterial activity (18). Some bacteria rely on hemin iron-containing molecules as a source of iron, such as hemin or hemoglobin (19). Hereby Fe^{2+} is removed from its carrier molecule, which may result in highly fluorescing metal-free porphyrin derivatives, i.e. protoporphyrin. Another source may be chlorophyll, whereby the Mg^{2+} is removed. To date the mechanism for porphyrin accumulation in carious dental hard tissue and calculus is not proven, but presence of small traces of blood from sulcus fluid and gingival margin and traces of blood and chlorophyll from the nutrition may be important sources for porphyrin derivatives.

Near-infrared fluorescence excited by 655 nm laser irradiation has shown feasibility for clinical subgingival calculus detection already (6, 7). The device used in these studies (Diagnodent®) excites at 655 nm and measures integral emission in the near-infrared spectral region. From the results of the present study it can be seen that excitation in the 400–420 nm range produces specific fluorescence in the red spectral region in dental calculus not present in clean root surfaces. Based on these data, optical calculus detection seems possible, but detection algorithms have to be developed that best distinguish between clean root surfaces and dental calculus similar to approaches that have been made with enamel carious lesions (20, 21).

This paper focused primarily on qualitative fluorescence properties of dental calculus. We could not find a correlation between thickness of calculus and fluorescence signal intensity, which may be due to the high optical density of calculus. This means that excitation light could hardly penetrate deeper layers so that the fluorescence signal had its origin mainly in the outer layers of calculus irrespective of calculus thickness. On the other hand it should be possible to evaluate the spatial distribution of calculus across, for example, the root surface with an appropriate instrument, i.e. a small fiberoptic probe.

No differences between supragingival and subgingival calculus were found in the present study. Hence, it does not seem to be possible to distinguish between supra- and subgingival calculus with fluorescence detection methods to date. Beyond its application for the detection of subgingival calculus within traditional periodontal treatment regimens, fluorescence calculus detection may become an integral part of periodontal treatment when coupled with laser- or light-based treatment technologies. Such coupling would allow for treatment of diseased root surfaces controlled by simultaneous detection.

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