Concomitant caries and calculus formation from \textit{in situ} dentin caries model [version 1; peer review: 2 approved]

Frederico B de Sousa$^1$, Pablo J Mangueira$^1$, David R Tames$^2$, Sandra S Vianna$^3$, Neriede S Santos-Magalhaes$^4$

$^1$Departmento de Morfologia, Centro de Ciências da Saúde, Universidade Federal da Paraíba, Cidade Universitária, 58051-900, João Pessoa, Paraíba, Brazil
$^2$Faculdade de Odontologia, Universidade do Vale do Itajaí, Itajaí, 88302-202, Santa Catarina, Brazil
$^3$Departamento de Física, Centro de Ciências Exatas e da Natureza, Universidade Federal de Pernambuco, Cidade Universitária, 50670-901, Recife, Pernambuco, Brazil
$^4$Laboratório de Imunopatologia Keizo-Asami (LIKA), Centro de Ciências Biológicas, Cidade Universitária, 50670-901, Recife, Pernambuco, Brazil

**Abstract**

The aim of this study was to test the possibility of the concomitant formation of calculus deposits and caries from \textit{in situ} dentin caries model for short time periods. Six volunteers wore palatal removal appliances with four polished dentin specimens protected from intraoral mechanical forces for up to 14 days. Each volunteer applied a 50\% sucrose solution (four times a day) on the specimens and performed a daily mouthwash with 0.05\% NaF. Samples were removed after 2, 5, 9 and 14 days \textit{in situ}. Demineralization was analyzed by stereomicroscopy and SEM (secondary electrons and backscattered electrons modes) and calculus was analyzed by energy dispersive spectroscopy and fluorescence spectroscopy. Seventeen samples, at least one sample from each volunteer, presented dental calculus on both carious and non-carious ones, detected in all time intervals. Ca/P ratios of dental calculus ranged from 1.1 to 1.7. Some large calculus deposits on carious surfaces were confirmed by fluorescence. In conclusion, concomitant caries and calculus formation can be found in dentin caries formed \textit{in situ}. This has important repercussions on the study of surface phenomena on the interface between hard dental tissues and dental plaque.

**Keywords**
dental caries, dental calculus, caries model, in situ, surface reactions

**Open Peer Review**

Approval Status  

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**version 1**  

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Introduction

*In situ* caries models are not expected to give rise to calculus formation. However, the formation of loosely mineralized deposits in dental plaque concomitantly with carious dissolution of enamel, in an environment with fluoridated mineralizing solution, has been reported. In a morphological study with transmission electron microscopy, those deposits were shown not to be identical to calculus as they were never present within intact bacterial cells. In addition, it was shown by scanning electron microscopy (SEM) that calculus (firmly bound to hard dental tissue) could develop on dentin specimens subjected to an *in situ* carious attack with a daily application of a 50% sucrose solution in individuals living in a water-fluoridated area for periods of four weeks. While demineralization is likely in such models, no clear-cut evidence was presented. In this study, some samples with calculus had the cariogenic challenge disturbed by weekly mechanical plaque removal, raising the hypothesis that the concomitant development of caries and calculus may occur during shorter periods under the given conditions.

The aim of this study is to report concomitant formation of calculus deposits and caries on dentin surfaces submitted to an *in situ* caries model (using sucrose and fluoride) for short time periods (2–14 days).

Material and methods

**Experimental design**

Human dentin specimens (3 × 3 mm) from non-erupted third molars, previously autoclaved, had their surfaces consecutively polished with slurries of decreasing grain size until the alumina was 1 mm. Then, they were mounted in customized hand-made resinous removable palatal appliances (thin resinous removable plates placed on the hard palate only and extending from the area close to the central incisors to area close to the first molars), each one containing four specimens distributed in two pairs each in a different side of the appliance, where they were protected from intra-oral mechanical forces by means of plastic perforated tape. A space of up to 2 mm was left between the specimen and the tape. In the mouth, specimens were located near the palatal surfaces of the first permanent molar and the first premolar.

Six volunteers, aged 20–29 years, were selected on the basis of the following criteria: (i) to be otherwise healthy (no medication regimen), (ii) no calculus treatment in the last two years and no clinical calculus deposits at the time of the study, and (iii) to present normal stimulated salivary flow. All volunteers were dental students who lived in non-water-fluoridated areas, and were given fluoridated dentifrices (1500 ppm of the same brand) daily. All volunteers provided signed informed consent. Ethical approval was obtained from the Universidade do Vale do Itajai Human Research Ethics Committee.

Intra-oral periods lasted fourteen days and the volunteers were instructed to use the appliances all day long, except during meals. Two drops of a 50% sucrose solution (the same applied previously) was applied onto the specimens only, four times a day to mimic meals, and a 0.05% NaF solution was provided to perform one daily mouthwash for about one minute with the appliance in the mouth (at a different time to the sucrose rinsing). Instructions were given to replace the appliance in the mouth just after the sucrose rinsing and to not brush on the specimens. For each volunteer, specimens were removed after 2, 5, 9 and 14 days of intra-oral period. Six other polished specimens prepared in the same way described above, not exposed to the oral cavity, were also selected for reproducibility during morphological analysis, to make a total of 30 specimens. After the *in situ* period, specimens were stored in 0.1% timol prior to further analysis.

**Scanning electron microscopy (SEM) and energy-dispersive spectroscopy (EDS)**

All samples were treated with NaOCl 5% for 15 minutes, to remove organic coatings, then critical point dried with CO₂-atmosphere of 10⁻² Torr and coated with a 10 nm-thick layer of gold. The secondary electron detector of a scanning electron microscope JEOL 5900HV (voltage of 15 KeV) was used. Each specimen was analyzed under tilting angles of 0° and 40°. EDS analyses were performed in representative areas by a VOYAGER system attached to the microscope using the following parameters: spatial resolution of 1 mm, electron beam area of 10 mm, working distance of 8–9 mm, voltage of 15 KeV and a live time of 60 s. Ca/P (mol/mol) ratios (mean of 5 measurements) were analyzed using a standardless method-intensity of the standard calculated on the basis of the spectral properties of the sample—with corrections for atomic number, absorption and fluorescence (ZAF correction). SEM analysis of surface changes (demineralization and dental calculus) was performed twice by blinded examiners with a time interval of seven days between the two analyses. A set of 3 images from each sample was chosen. The Kappa test was used to calculate agreement. Only Schroeder’s type A dental calculus mineralization centers (with mineralized bacterial bodies outlines) were considered.

**Stereomicroscopy**

After SEM, all samples were treated with NaOCl 5% for 15 minutes to remove the gold coating and then hemi-sectioned centrally on the exposed surface. The surfaces created after cutting were gently polished with alumina 10 mm and then analyzed under the stereomicroscope (Leica MZ12, Leica, Switzerland). Image analysis for the automated identification of demineralized areas and lesion depth measurements were performed using Leica QWIN Plus software (Leica, Switzerland). A standard margin of color discrepancy detection in the software was used for all samples when the software was asked to differentiate sound and carious dentin. The only influence of the operator was to choose a 1 mm-wide central area for automated lesion depth measurement.

**Fluorescence spectroscopy and backscattered scanning electron microscopy (BSE-SEM)**

In samples that presented thick (> 100 μm in height) and extensive calcified deposits, a scalpel was used to remove the surface by scraping after stereomicroscopy. The scraped material was dissolved in a solution of HCl 27% (~ 0.2 ml), dispersed in a quartz cuvette and submitted to fluorescence spectroscopy in a commercial photon-counting spectrophluorometer (PC1, ISI, USA and Vinci software, ISI, USA) equipped with a xenon arc lamp operating at 10 mA, using 1 mm slits (bandwidth of 8 nm). Excitation wavelength (λ<sub>exc</sub>) was 416 nm and the emission (λ<sub>em</sub>) was collected from 425 to 800 nm (mean of 15 readings). Fluorescence of the other two saturated solutions in HCl 27%, one with human dental calculus and another with human dentin, were also analyzed.
Occurrence of demineralization and calculus deposits was depicted from SEM examination (Table 1). Kappa’s coefficients of intra-examiner agreement were 0.88 and 0.83, and 0.78 for inter-examiner agreement of the diagnosis of demineralization with SEM. For dental calculus, it was decided to count only cases with full agreement between examiners. Mean lesion depth values were 0.0, 0.197 (± 0.059), 0.442 (± 0.062) and 1.100 mm (± 0.212) for 2, 5, 9 and 14 in situ periods, respectively (differences were statistically significant, p < 0.05). BSE-SEM analysis confirmed the presence of demineralization (lower gray levels due to lower mean atomic number) detected by stereomicroscopy.

Seventeen samples presented calculus deposits (14 with demineralization; Table 1). Seven of these presented ~ 50% or more of the surface area covered by the deposits. In the other samples, deposits formed isolated or connected islands of 50–500 mm wide. Ca/P ratios resemble the structures of dicalcium phosphate (Ca/P of 1.0), octacalcium phosphate (Ca/P of 1.3), magnesium whitlockite (Ca/P of 1.5) and hydroxyapatite (Ca/P of 1.67–2.3) (Table 1). Mean Ca/P ratio of dentin (for all samples) was 1.88 mol/mol (± 0.22), compatible with hydroxyapatite. Demineralization surrounding calculus deposits occurred in thirteen samples.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Demineralization</th>
<th>Dental calculus (Ca/P, mol/mol)</th>
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<tr>
<td>I</td>
<td>5, 9 and 14 days</td>
<td>5 days (1.04 ± 0.03)</td>
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<tr>
<td>II</td>
<td>2, 5, 9 and 14</td>
<td>2 (1.12 ± 0.04), 9 (1.40 ± 0.02) and 14* days (1.53 ± 0.04)</td>
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<tr>
<td>III</td>
<td>5, 9 and 14 days</td>
<td>2 (1.49 ± 0.07), 5 (1.48 ± 0.05) and 9* days (1.43 ± 0.06)</td>
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<tr>
<td>IV</td>
<td>5, 9 and 14 days</td>
<td>2 (1.47 ± 0.02), 5* (1.52 ± 0.04), 9* (1.66 ± 0.06) and 14 days (1.73 ± 0.05)</td>
</tr>
<tr>
<td>V</td>
<td>5, 9 and 14 days</td>
<td>2 (1.38 ± 0.02), 5 (1.51 ± 0.05), 9 (1.54 ± 0.05) and 14* (1.71 ± 0.06) days</td>
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<tr>
<td>VI</td>
<td>2, 5, 9 and 14</td>
<td>5 (1.56 ± 0.05) and 14* (1.41 ± 0.04) days</td>
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Crystallite morphology and Ca/P ratios resembling hydroxyapatite in 10-day old calculus deposits have been reported previously. As the in situ period proceeded, calculus deposits (resembling the morphology of Schroeder’s type A calculus deposits) with increased height, macroscopic in some cases, were seen on demineralized areas (Figure 1A–C). The shapes of intact bacterial bodies (coco, filaments and rods) remained within extracellular calcified trabeculae, indicating that the mineralization process was preferably extracellular. Some deposits were identified only after tilting the sample to 40°. No bacterial cell remnants were identified within calculus deposits.

Fluorescence of human dental calculus in acid solution has been reported to present the highest emission with λ_{nuc} at 416 nm and λ_{min} peaks at 620 and 660 nm (originated from hematoporphyrin). A lower fluorescence emission band below 580 nm has also been reported for human dental calculus. Human dentin fluoresces with a λ_{min} peak at 440 nm and does not present any emissions in the region of 580–700 nm, which is in accordance with our fluorescence data for λ_{nuc} at 416 nm (Figure 1F). The selected samples with “large” calcified deposits (deposits < 100 µm in height cannot be detected by a spectrophotometer system with a lamp source as excitation light) showed a mixture of fluorescence bands of human dental calculus and human dentin (Figure 1F). The double treatment with NaOCl 5% and the absence of bacterial cell

The differences between in situ periods of all volunteers with regard to lesion depth were evaluated by paired t test (significance limit at 5%).

Table 1. Occurrence of demineralization and calculus deposits (with mean of 5 Ca/P ratios obtained by EDS-SEM). Samples with 50% or more of their surfaces covered by calculus.
remnants during SEM examination exclude the influence of loosely bound organic material of bacteria on the observed fluorescence.

The concomitant development (based on our time intervals) of caries lesions and calculus deposits using an in situ caries model reported here occurred under conditions known to cause the formation and growth of dental plaque. Combining SEM, fluorescence spectroscopy and stereomicroscopy (Figure 1A–F), we have shown caries and calculus developments in the same dentin sample that was originally non-carious and had not been exposed to the oral cavity before the in situ experiment. Our model was able to promote time-dependent demineralisation (seen from lesion depth data), which means that volunteers followed instructions properly. To our knowledge, this is the first time that this phenomenon has been reported on the basis of the combination of such mutually validating techniques.

Conclusion
In conclusion, calculus formation in active cariogenic dental plaque has important repercussions on the study of surface phenomena on the interface between hard dental tissues and dental plaque. It is also important in the development and the evaluation of anti-tartar and caries-preventative agents. Our study shows that more attention should be paid by those studying in situ dentin caries to the identification of calculus on hard dental tissues exposed to in situ caries models.

Author contributions
Sousa FB and Tames DR designed the study and the intra-oral appliances. Mangueira PJ selected volunteers, prepared intra-oral appliances, and dentin samples. Sousa FB and Mangueira PJ conducted the in situ protocols and followed up with volunteers. Sousa FB, Vianna SS and Santos-Magalhaes NS conducted the fluorescence spectroscopy, SEM and BSE analysis, and statistical analysis. Sousa FB and Tames DR wrote the paper. All authors read and approved the final manuscript.

Competing interests
No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Consent
All volunteers who used the intra-oral appliances signed a written informed consent before participating in this study. No payment was provided to volunteers in return for their participation in this study.

References

It was noted that all dentin samples were exposed to sucrose solutions, fluoride and were protected by tape. Only the unexposed slabs were used as controls and none of these three conditions were controlled. A more complete picture would have been possible with their inclusion.

The authors claimed “It is known that dicalcium phosphate and octacalcium phosphate can be precipitated in acidic environments whereas hydroxyapatite is dissolved.” They listed references 7 and 8 to support this statement. However, I was unable to access reference 7 and could not find supporting data in reference 8. While DCP is slightly less soluble than HAP around pH4 I would not consider that to mean that either DCP or OCP readily precipitate at that pH.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
lesions with known history and with severity directly proportional to the in situ time. Mean lesion depth increased with in situ time, confirming the prediction. In an environment where caries develops, surface features of the samples were analyzed in order to verify whether calculus deposits could have been formed under the same conditions. Such conditions are those common to in situ caries models. Because the length of the article is short, we focused on the more relevant data (samples with dental calculus). If we had exposed dentin samples that remained sound after the in situ period, the presence of absence of calculus deposits on those samples would have not contributed to prove that calculus deposits can be formed concomitantly with caries lesions in short time intervals in an in situ caries model.

In the case of precipitation of dicalcium phosphate (DCP) and octacalcium phosphate (OCP) in acidic environments, Johnsson et al. 1992 describes evidence indicating that formation of both DCP and OCP in vivo, during normal mineralization, is quite different from the conditions operating under pathological mineralization. The authors cite reports of the precipitation of DCP and OCP in slightly acidic conditions (please see last paragraph of the second column on page 78 of Johnsson et al. 1992). The critical pH of dentin is between 6.5 and 7 (Hoppenbrouwers et al. 1987). There is a pH range below the critical pH of dentin that can be considered as slightly acidic, and so it is consistent with the possible precipitation of both DCP and OCP in our samples. Regarding formation of DCP and OCP in acidic conditions in vitro, evidence can be found in the following papers; De Rooij et al. 1984 and Lundager Madsen et al. 1991.

**Competing Interests:** This comment was posted by the authors of the study.

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Leonard A. Crocombe

Australian Research Centre for Population Oral Health and the School of Dentistry, The University of Adelaide, Adelaide, Australia

**Competing Interests:** No competing interests were disclosed.

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