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Yoshihiko Hayashi
Kyushu University

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HIGH RESOLUTION ELECTRON MICROSCOPY OF THE JUNCTION BETWEEN ENAMEL AND DENTAL CALCULUS

Yoshihiko Hayashi

Department of Conservative Dentistry,
Faculty of Dentistry, Kyushu University 61,
Maidashi 3-1-1, Fukuoka 812, Japan

Phone No.: 92-641-1151 Ext.5153 FAX No.: 92-641-8027

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Abstract

Newly formed dental calculus grows on the enamel surface after the tooth crown is exposed to the oral environment. In order to demonstrate the crystal coherence, the junction between enamel and dental calculus was examined in the high resolution electron microscope. Ultrathin sections were also used for selected area electron diffraction to reveal any newly formed mineral phase.

High resolution electron microscopy (HREM) revealed that lattice fringes of dental calculus crystals directly coincided with those of enamel crystals. Two types of coherence were identified at the junction: in one type, a dental calculus crystal contacted the side of an enamel crystal; in the other type, the former contacted the tip of the latter. Tilt boundaries between lattice striations showed varying degrees of curvatures in contact levels. A dislocation of lattice striations was also observed at the junctional regions. Selected area electron diffraction patterns of dental calculus were almost consistent with hydroxyapatite (OH-AP).

These findings indicate that the elongation and/or enlargement of lattice fringes of calculus crystals during their growth brings direct coherence with enamel crystals. This suggestion is supported by the fact that it is clinically difficult to completely remove the dental calculus without the loss of the superficial layer of enamel.

Key Words: Dental calculus, enamel, initial calculus formation, high resolution electron microscopy, selected area electron diffraction, hydroxyapatite, crystal plane, lattice fringes, contact, dislocation.

Introduction

Using a transmission electron microscope (TEM), Gonzales and Sognnaes [7] first reported that dental calculus consisted of densely mineralized areas entrapping many degenerating microorganisms, within which were deposited electron-dense needle-like crystals. As dental calculus is always covered by an unmineralized dental plaque layer, it may be difficult to estimate whether calculus itself has a harmful effect during periodontal disease. However, its presence makes adequate plaque removal almost impossible for the therapist, and prevents patients from performing an efficient plaque control [24]. Following this, a comprehensive understanding of calculus attachment to the tooth should be examined and studied by the clinicians attempting complete and precise removal [1]. In the 1970s, electron microscopy revealed that the dental calculus crystals came into intimate contact with enamel, cementum or dentine crystals [1, 22]; however, a direct crystal coherence between the two deposits has not yet been demonstrated.

Recently, lattice fringes and atomic structures in synthetic apatites and enamel crystals have been observed in the modern high resolution TEM (HREM) [15, 17-19], and the fusion of lattice fringes in the dentino-enamel junction has been also demonstrated using a similar technique [11]. The present study was designed to investigate possible direct crystal coherence at the junction between dental calculus and enamel using a HREM operating at an accelerating voltage of 300 kV.

Materials and Methods

A human mandibular impacted third molar of a 58-year-old man was used. Its occlusal plane was partially exposed to the oral cavity. Pieces of occlusal enamel about 2 mm in width were excised with a diamond disk under a stream of Ringer's solution. They were immersed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 for 24 hours, postfixed in 2% osmium tetroxide in the same buffer for 1 hour, and then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Ultrathin sections were cut with a MT-5000 ultramicrotome

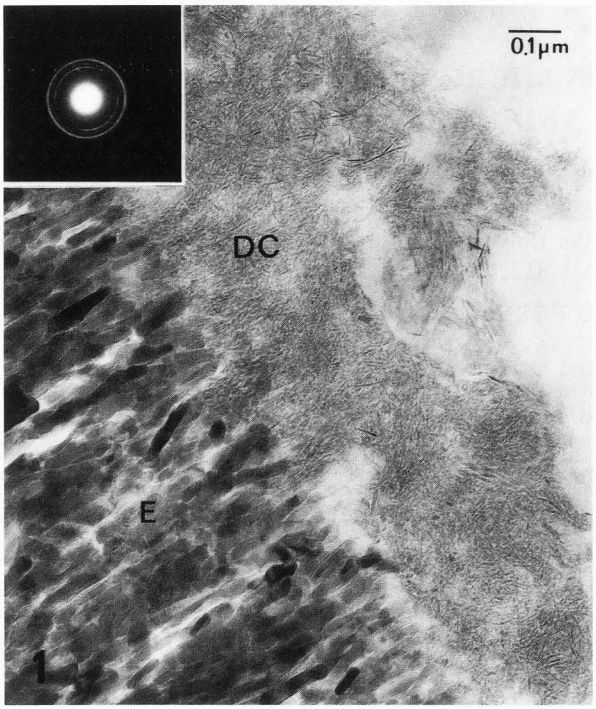

Figure 1 (at left). The junction between enamel (E) and dental calculus (DC), calculus crystals are in intimate contact with the enamel crystals. Inset: The electron diffraction pattern of calculus crystals. Note the concentric inner double rings characteristic of apatite.

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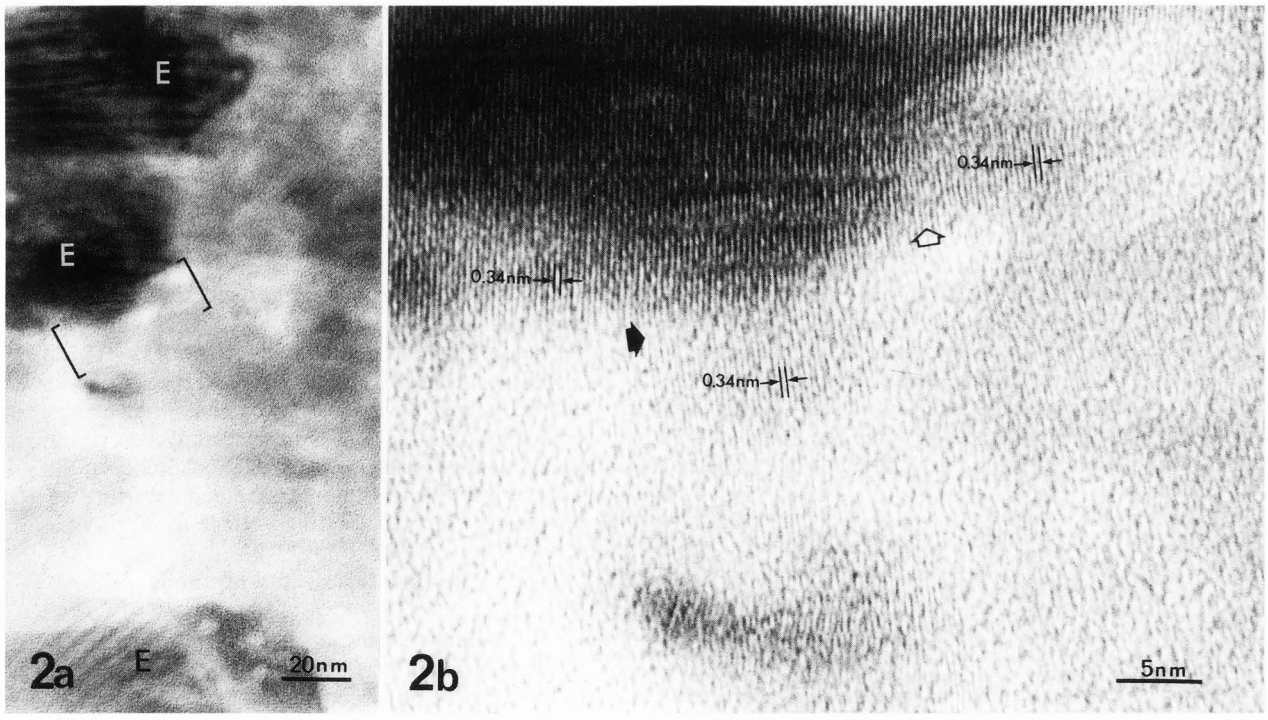
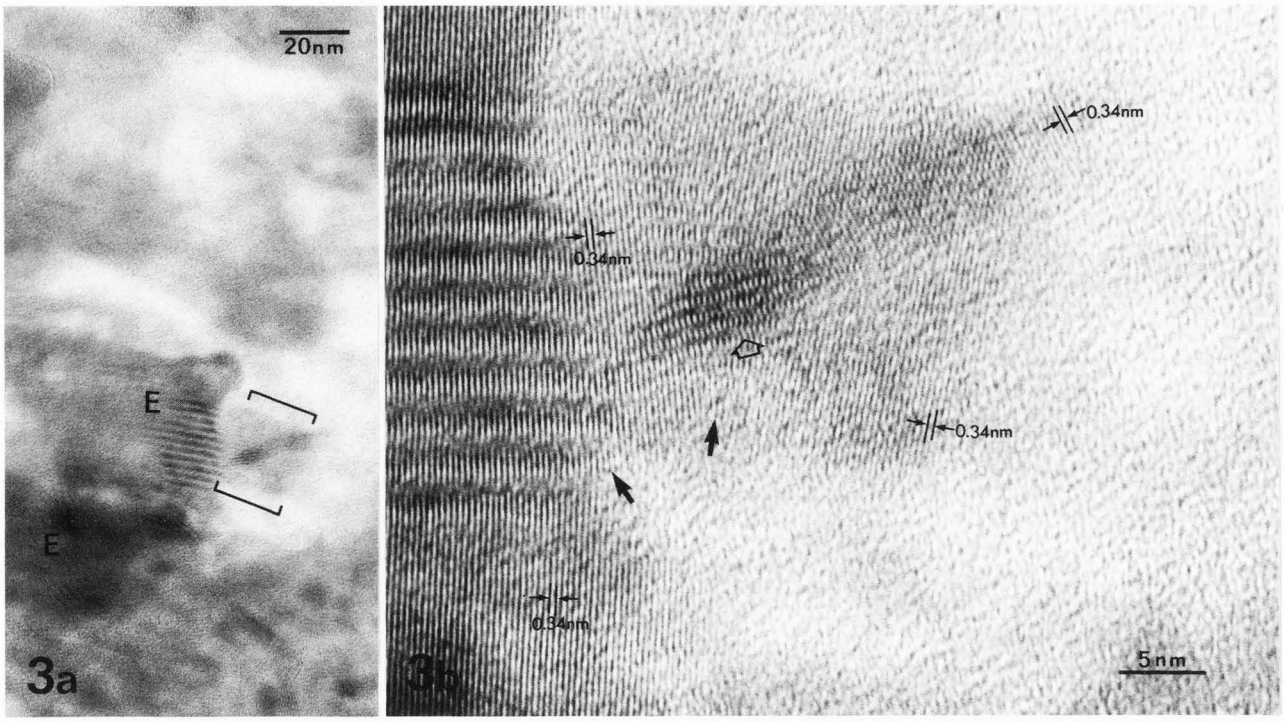
equipped with a diamond knife and collected in a specimen boat containing a saturated solution of human dentine powder to prevent demineralization of the sections [10]. Ultrathin sections were prepared as parallel to the c-axis of enamel crystals as possible. They were mounted on Formvar-coated 200-mesh copper grids reinforced with carbon about 5 nm in thickness and examined without staining in a Hitachi H-9000 electron microscope at an accelerating voltage of 300 kV. The 300 kV microscope was fitted with a C_s = 2.5 mm objective pole piece, and had a lattice resolution of 0.14 nm and a point-to-point resolution of 0.25 nm. After the junction between enamel and dental calculus was confirmed at a magnification of 30,000x, HREM was carried out at a direct magnification of 300,000x. A liquid nitrogen-cooled anti-contamination device was used during the specimen observation.



Figures 2a,b. High resolution electron micrographs from the junction. **a)** High magnification image of the junction between enamel and dental calculus. Calculus crystals (bracket area) are just adjacent to enamel crystal (E). **b)** High resolution image of the contact area (bracket area in a). Lattice striations in calculus crystal, with 0.34 nm interval, contact with (002) fringes of enamel crystal at the side of an enamel crystal (large closed arrow). Lattice striations in calculus crystal, with 0.34 nm interval, contact with (002) fringes of enamel crystal at the tip of an enamel crystal (open arrow).

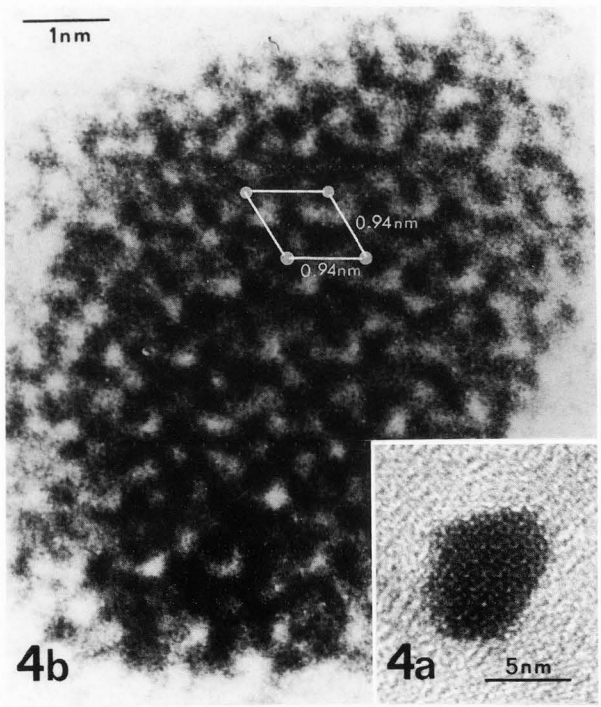


Figures 3a,b. High resolution electron micrographs of the more complex junctional region. **a)** High magnification image of the junction between enamel and dental calculus. Calculus crystal (bracket area) is just adjacent to enamel crystal. **b)** High resolution image of the contact area (bracket area in Fig. 3a). Two appositions of lattice striations (0.34 nm in interval) of enamel crystals are observed in Fig. 3b (large closed arrow). A tilt boundary is located at the dislocation level. The calculus crystal (about 5 nm in width) having (002) spacing is shown from upper right to lower left at the central part of this figure. Crystal contact between enamel and calculus is seen at the apposition level of enamel crystals (open arrow).

Furthermore, ultrathin sections were used for selected area electron diffraction to identify the mineral phase of dental calculus. D-spacings of diffraction patterns were calibrated from those of gold obtained under identical conditions.

Results

The enamel surface partially exposed to oral cavity was covered with extremely thin layer of dental calculus which could not be identified with the naked eye. The thickness of the calculus was about 7 μm in the thickest region. Enamel crystals ranging up to 40 nm in width could be easily distinguished from the smaller calculus crystals measuring 5 nm in width (Fig. 1). Selected area electron diffraction patterns were obtained from



Figures 4a,b. High resolution image of the (001) plane of a single calculus crystal. **a)** The periodic arrangement of black points is observed in the crystal plane. **b)** Enlarged image of a). A projection of the apatite unit cell is marked in white.

the dental calculus (Fig. 1 inset) and revealed a pattern of concentric inner double rings. These inner double rings had d-spacings (about 0.346 and 0.281 nm respectively) consistent with OH-AP (0.344 and 0.2814 nm).

The enamel crystals had an intimate association with the dental calculus crystals (Figs. 2 and 3). HREM revealed a direct coherence between lattice fringes in the enamel and calculus crystals. Two types of associations were observed at the junction (Fig. 2): one in which a dental calculus crystal contacted at the side of an enamel crystal; the other in which the former contacted at the tip of the latter. In the present study, the contact between the (002) spacings having 0.34 nm dimensions was observed in the calculus and enamel crystal in both types of associations. Many Moiré patterns were observed near and at the interface where the dislocation appeared to form tilt boundaries.

A more complex association between dental calculus and enamel is indicated in Fig. 3. In this case, the apposition of well-defined lattice fringes {the (002) spacings} of an enamel crystal could be seen at two dislocation levels where intricate and irregular tilt boundaries were located and a curvature of lattice fringes was also observed. Dental calculus, the lattice fringes of which also corresponded to the (002) spacings having 0.34 nm dimensions, contacted with the enamel crystal at just this apposition level between enamel crystals. Moiré patterns were observed at the contact level where a curvature of lattice fringes was clearly identified.

The near atomic image of a single calculus crystal plane is shown in Fig. 4. The diamond shape (each side: 0.94 nm) was identified in this plane. Each point in the corners of the diamond shape was 0.3 nm in diameter.

Discussion

Dental calculus observed in the present study is thought to be a very early one according to both macroscopic and microscopic findings. As the enamel surface on which dental calculus deposits is ultrastructurally not smooth, it might become a suitable scaffolding to mechanically lock the calculus crystals observed at the surface of dentine and cementum [1, 22].

The enamel surface first exposed to the oral environment was immediately covered by pellicle originating from saliva. The innermost layer of calculus where the direct connection between enamel and calculus crystals was observed is thought to be originally occupied by pellicle. Then, the mineralizing mechanism of the innermost layer of calculus not containing microorganisms must be distinguished from that of outer layer of calculus containing microorganisms.

Saliva is a metastable solution for some of calcium phosphates, namely apatite, octacalcium phosphate (OCP), and whitlockite. This means that saliva is supersaturated with respect to these salts and able to support crystal growth, however a spontaneous precipitation does not occur without the presence of crystals on which new crystals can form [24, p.120]. Crystals for this nucleation process are present in the enamel surface, but since that surface is generally covered by pellicle, it is not easily available for this function. It is suggested that the protease in saliva might play important role in the mineralization of dental calculus [16, 25]. On the other hand, statherin [20] and proline-rich protein [9] have been reported as the inhibitors of calcium phosphate precipitation in human saliva. The degradation of these proteins results in complete loss of the activity [8]. Furthermore, some of the products of proteolysis including ammonia and amines (especially ammonia) increase plaque pH and enhance calculus formation [5]. Proteolytic degradation by oral bacteria is one of the possible mechanisms for the loss of inhibitory activity. Many oral bacteria produce proteolytic enzymes [6] and dental plaque also shows proteolytic activity [23]. These reports suggest that proteases could degrade peptides of pellicle and enhance the spontaneous calcium phosphate precipitation, and microorganisms indirectly contribute for the initial calcium phosphate deposition on enamel surface. After that, the acidic phospholipids in the membrane of microorganisms are thought to provide sites for initial Ca^{2+}-binding followed by calculus growth [3, 4].

The mechanism of contact between enamel and calculus is subsequently of interest: which grow and contact to the other? When the enamel surface is first exposed to the oral cavity, a thin layer of pellicle originating from saliva immediately covers the surface. Dental calculus formation starts with deposition of calcium phosphate within this thin layer. HREM revealed that lattice fringes of calculus crystals coincided with those of the enamel crystals. Furthermore, the tooth examined in this study was the adult third molar whose enamel crystals are thought to be fully developed. These findings strongly suggest that growth of calculus crystals occurred in direct contact with enamel crystals. This is also supported by the fact that it is very difficult to completely remove dental calculus from enamel surface.

Although selected area electron diffraction patterns obtained from early dental calculus were the most consistent with OH-AP, other phases such as brushite, whitlockite, and OCP have been revealed in X-ray diffraction analyses [12, 21] and energy-dispersive X-ray microanalysis [14]. These other phases have similar electron diffraction patterns to OH-AP, especially at the d-spacings of around 0.34 and 0.28 nm dimensions. Furthermore, it is thought that in some cases, they are unstable in the electron beam. These findings suggest that the early phase of calculus crystals might mainly consist of apatite but that the existence of other phases would be undeniable in the present study.

Each point in the corners of the diamond shape of a single calculus crystal plane was 0.3 nm in diameter consistent with hydrogen atom (0.308 nm) [26]. Polarized infra-red [2] and neutron diffraction [13] results in OH-AP confirmed that oxygen-hydrogen bond direction was parallel to the c-axis and that the distance of oxygen-hydrogen was 0.1 nm. Furthermore, the oxygen

atom was 0.264 nm in diameter [26] and ultrathin sections in the present study were 80 nm in thickness. These findings indicate that the diamond shape is identified as the unit cell of the (001) plane on OH-AP [27] and that oxygen atoms are overlapped by hydrogen atoms in the (001) plane. Then, these points located in the corners of the unit cell are thought to correspond to the OH ions.

Crystallographically, the structure of fluorapatite (F-AP) is very similar to that of OH-AP. The possibility of the discrimination between OH-AP and F-AP has been considered using the high resolution (001) plane. The a-axis in the unit cell of OH-AP and F-AP was determined by X-ray diffraction and neutron diffraction analyses as 0.9422 nm and 0.9364 nm respectively [27]. These analytical data suggest that it seems difficult to distinguish OH-AP from F-AP on the a-axis because the difference is theoretically only 87 μm on the enlarged image at the magnification of 15,000,000x. The diameter of each point located in the corners of the unit cell was 0.3 nm consistent with OH ion (0.308 nm) [26]. On the other hand, the F ion was 0.266 nm in diameter [26]. The difference in diameter between OH and F ions is theoretically 630 μm on the enlarged image at the magnification of 15,000,000x. These findings indicate that OH-AP could be distinguished from F-AP on the near atomic image of a single calculus crystal plane.

In conclusion, dental calculus crystals formed on enamel surface rigidly and intimately locked together with enamel crystals. HREM revealed that sets of lattice striations in early dental calculus crystals directly coincided with those of enamel crystals. Furthermore, present methods for HREM are thought to be useful to clarify the crystal contacts and dislocations at the subcrystal level in the field of mineralized tissues.

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Discussion with Reviewers

M. Goldberg: When dental surgeons try to remove calculus during prophylaxis, does the author believe that part of the enamel located near the surface is also removed, or would he suggest that the deepest part of the calculus is always left? The clinician implication of the paper is that attempts to remove calculus are either dangerous or not useful.
Author: The superficial layer of enamel is removed during scaling. After scaling, we polish the surface (root planing) to smooth it. Then, the present HREM demonstrates that the root planing is indispensable after scaling.

D.G.A. Nelson: What crystal growth mechanism might occur to cause calculus crystals to align in the same crystallographic direction as the enamel apatite crystals?
Author: Crystal growth occurs through so-called additional mineralization. It is favorable and reasonable for getting the large contact area that calculus crystals align in the same direction as the enamel ones.

D.G.A. Nelson: What causes the apparent change in density between the calculus and enamel regions? Are the calculus crystals packed less densely, are they thinner, or is it an artifact of the sectioning process?
Author: Enamel crystals are harder than calculus ones. Calculus crystals are thought to be equally sectioned in comparison with enamel ones. As all micrographs are taken without staining for EM contrast enhancement, the electron density reveals pure inorganic structures. Then, these indicate that the change in density between the calculus and enamel regions is probably due to the difference of a crystallinity.

E. Bonucci: Where do early calculus crystals form? Are they nucleated on the surface crystals of enamel, or might they be formed in connection with organic component(s) of protease-modified saliva pellicle, to successively grow coherently with the enamel crystals, or are there other possibilities?
Author: Apart from this paper, the enamel surface has been extensively examined by me. I could observe the initial fine crystallites having lattice fringes just over the enamel surface (about 5 nm at a distance). However, I have not yet investigated the connection with organic components.

Reviewer V: Since low magnification micrographs are not presented, how can one be certain that this was indeed calculus?
Author: I have confirmed the continuity from the early mineral deposit on the enamel surface to the mineral deposit containing microorganisms.

Reviewer V: Although this is a thorough study of only one tooth, we are concerned that one sample is not enough. Please comment.
Author: Three blocks from one tooth were thoroughly studied. The early stage of dental calculus is easy to get ultrathin sections and is thought to be suitable to observe the intact junction between enamel and calculus crystals. As the occlusal plane used in this study is partially exposed to the oral cavity, the situation might be similar to fully erupted teeth.