Scanning Microscopy

Volume 9 | Number 2 Article 11

5-28-1995

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Scanning Microscopy, Vol. 9, No. 2, 1995 (Pages 419-427) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

HIGH RESOLUTION ELECTRON MICROSCOPY OF THE INTERFACE BETWEEN DENTAL CALCULUS AND DENTURE RESIN

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(Received for publication December 22, 1994 and in revised form May 28, 1995)

Abstract

Dental calculus may grow on the denture surface. In order to demonstrate the mechanism of deposition, the interface between calculus and denture resin was investigated using a high resolution electron microscope. Ultrathin sections were also used for electron diffraction of selected areas to reveal any mineral phase.

The mineral layers without mineralized bacteria adjacent to the denture surface revealed a marked variation in thickness and crystal shape. Three types of crystal shape were observed at the junction: needle-like, rod-like and plate-like crystals. High resolution electron microscopy (HREM) showed that both rod-like and plate-like crystals were an aggregation of fine crystallites. The lattice fringes of the fine crystallites were observed among the near atomic structures of resin polymer at the interface in all three types of crystals. The electron diffraction patterns of selected areas revealed that needle-like and rod-like crystals were composed of hydroxyapatite (OH-AP), while plate-like crystals were composed of a mixture of OH-AP and whitlockite.

These findings indicate that, after saliva penetrates through the acrylic resin, calcium and phosphate ions in the saliva are trapped in the molecular chains of the resin polymer, while the local ion concentration then increases to reach supersaturation, whereas a spontaneous precipitation would occur at the superficial layer of the denture resin. Furthermore, a thin intermediate layer of crystallites might be indispensable for the scaffolding process in the calculus formation on the denture surface.

Key Words: Mineral deposits, denture, acrylic resin, dental calculus, high resolution electron microscopy, selected area electron diffraction, hydroxyapatite, whitlockite, interface, lattice fringes.

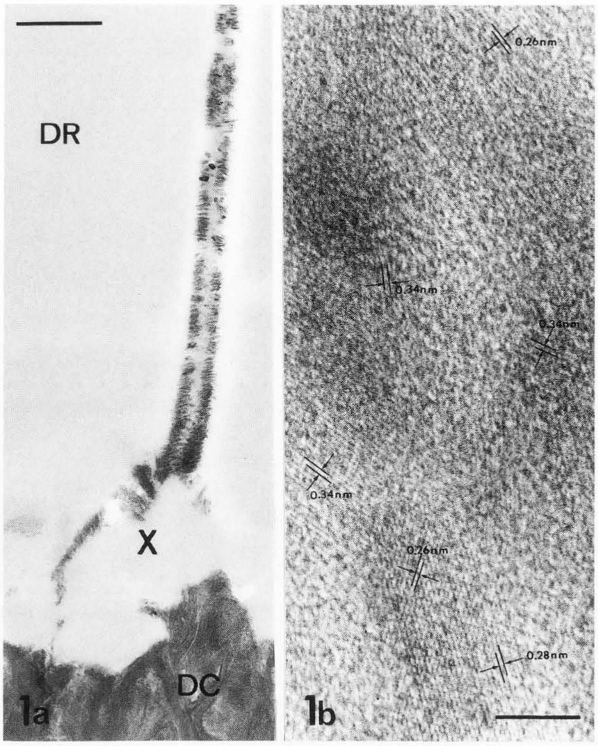
Introduction

Denture plaque on the acrylic resin contains microorganisms that can result in varying grades of denture-associated inflammations. Until now, most ultrastructural studies have focused on bacteria in the denture plaque associated with denture stomatitis [4, 5, 9, 32], while, in contrast, hardly any attention has been paid to mineral deposits adjacent to the denture resin. Recently, scanning electron microscopy and energy-dispersive X-ray microanalysis have revealed that early dental calculi on resin plates were composed of apatite, octacalcium phosphate (OCP) and brushite [22]. However, dental calculi composed of apatite and whitlockite have also been reported in studies using energy-dispersive spectroscopy [27]. Furthermore, the initial mineral deposition and crystallite contact at the junction in the dental calculus formation on the enamel surface have been investigated by high resolution electron microscopy (HREM) [13, 14]. Although dental calculus is often observed on the denture resin, the mechanism behind such mineral deposition on this organic material (resin polymer) has not yet been elucidated.

The present study was designed to ultrastructurally investigate the mechanism of mineral deposition on the denture resin. In particular, the possible contact at the junction between dental calculus and denture resin was examined using a high resolution electron microscope at an accelerating voltage of 300 kV.

Materials and Methods

A partial denture which had been discarded after the completion of a new denture was used in this study. It was immersed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 24 hours. After fixation, 3 pieces of the superficial layer of resin demonstrating brown deposits (2 mm in width) were excised from the lingual side of denture plate corresponding to the lower central artificial incisors with a diamond disk under a stream of Ringer's solution. They were postfixed in 2% osmium tetroxide in the same



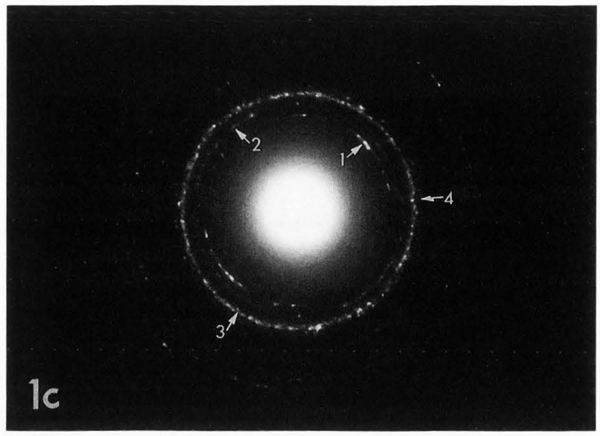


Figure 1. (**a**) A low magnification image of small rod-like crystals at the crack of the denture resin (DR). Dental calculus (DC) contains mineralized bacteria. X indicates the artificial space. Bar = 1 μm. (**b**) A high resolution image of a rod-like crystal. Note the aggregation of crystallites positioned in different directions. Bar = 5 nm. (**c**) Typical electron diffraction pattern of the region of rod-like crystals in Figure 1a. D-spacings of 0.343 (arrow 1), 0.307 (arrow 2), 0.280 (arrow 3) and 0.263 (arrow 4) nm correspond to the 002, 210, 211 and 202 indices of OH-AP.

buffer for 1 hour and then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin, without using propylene oxide in order to avoid any dissolution of the denture material. After polymerization, 10 specimens (about 1 mm x 1 mm x 1 mm), were re-embedded in epoxy resin. Ultrathin sections were cut with a MT-5000 ultramicrotome with a diamond knife and collected in a specimen boat containing a saturated solution of human dentine powder to prevent section demineralization [12]. They were mounted on Formvar-coated 300-mesh copper grids reinforced with carbon, about 5 nm in thickness, and examined without staining in a Hitachi H-9000 electron microscope at operated at an accelerating voltage of 300 kV.

Furthermore, the ultrathin sections were also used for electron diffraction of selected areas in order to identify the mineral phase of the dental calculus. The d-spacings of the diffraction patterns were calibrated from those of gold obtained under identical conditions.

Results

The denture surface was covered with a thin layer of dental calculus which was macroscopically recognized as the brown deposit. The thickness of the calculus was about 120 μm in the thickest region. The dental calculus consisted of two parts: the outer thick mineralized area entrapping many degenerated bacteria, within which needle-like crystallites were deposited, and the inner thin mineralized layer without any mineralized bacteria. The electron diffraction patterns of selected areas were obtained from the dental calculus (containing mineralized bacteria) consisting of needle-like crystallites; a pattern of concentric rings was revealed. These inner triple rings had d-spacings (about 0.343, 0.309 and 0.280 nm, respectively) corresponding to 002, 210 and 211 indices of OH-AP.

Although no mineralized structures were generally observed on the inside of the denture resin, small cracks containing mineral deposits were formed on the denture surface of two blocks (Fig. 1a). The depth of the crack was about 35 μm in the deepest region. The inside of the crack was filled with rod-like crystals, as seen at low magnification. HREM revealed that the rod-like crystals were composed of an aggregation of many fine crystallites positioned in different directions (Fig. 1b). The electron diffraction patterns of the selected areas obtained from the region of rod-like crystals revealed a pattern of concentric rings. Four rings had d-spacings of 0.343, 0.307, 0.280 and 0.263 nm, which corresponded to 002, 210, 211 and 202 indices of OH-AP (Fig. 1c).

The contact between the calculus and the denture surface was investigated using ultrathin sections without artificial spaces at the interface. Mineral deposits just

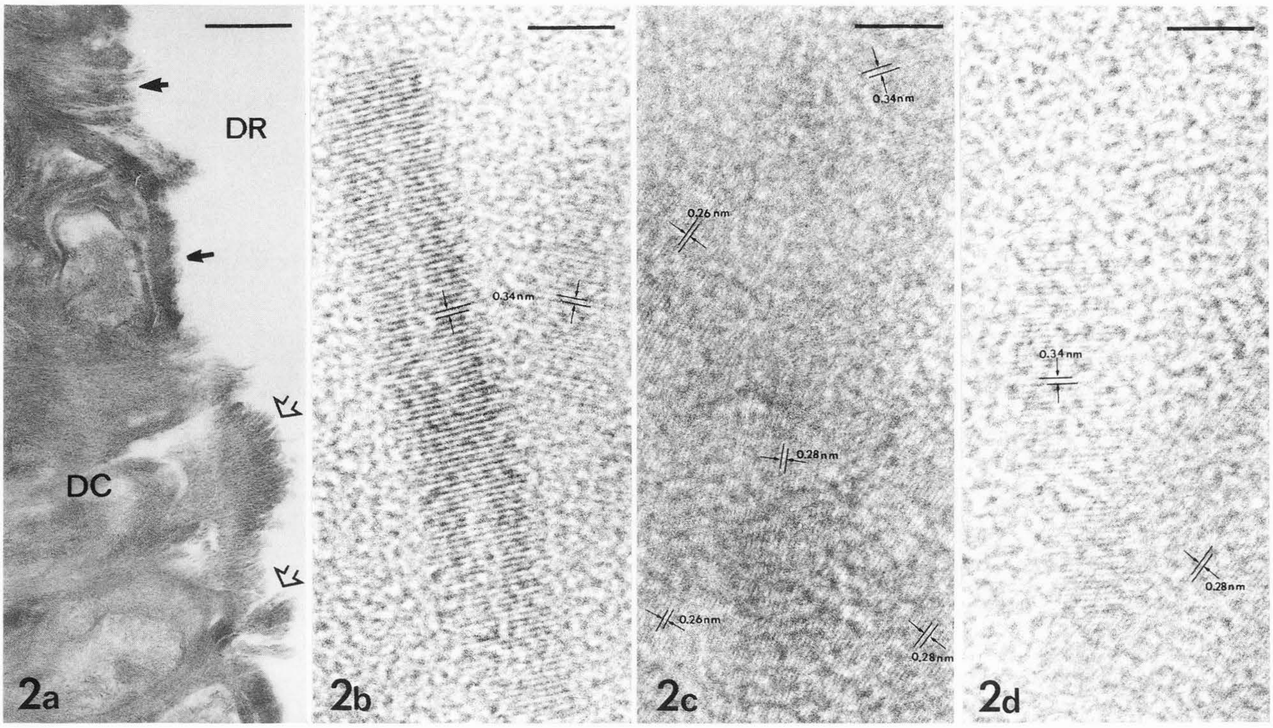


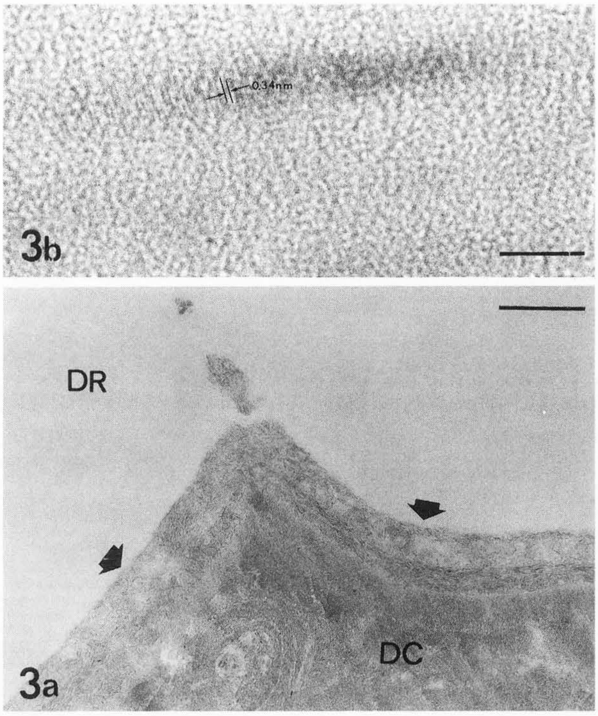
Figure 2. (**a**) A low magnification image of the interface be-tween dental calculus (DC) and denture resin (DR). Note the needle-like crystals (closed arrows) and rod-like crystals (open arrows) at the junction. Bar = 0.5 μm. (**b**) A high resolution image of needle-like crystallites at the interface. The lattice fringes of a fine crystallite come in contact with the granular structures of a resin polymer. Bar = 5 nm. (**c**) A high resolution image of a rod-like crystal. Note the aggregation of the crystallites positioned in different directions. Bar = 5 nm. (**d**) A high resolution image at the interface. The lattice fringes of a fine crystallite come in contact with the granular structures of the resin polymer. Bar = 5 nm.

on the denture surface revealed marked variations in the thickness and shape of the crystals. Three types of crystals were observed at the junction: needle-like, rod-like (Fig. 2a) and plate-like crystals.

HREM revealed that the lattice fringes of the needle-like crystallites came in contact with the near atomic structures of the acrylic resin polymer at the interface between the thin layer of needle-like crystallites adjacent to mineralized bacteria and the denture resin (Fig. 2b). HREM showed that the rod-like crystals consisted of an aggregation of many fine crystallites positioned in different directions (Fig. 2c), while the lattice fringes of rod-like crystals also came in contact with the near atomic images of acrylic resin polymer at the junction (Fig. 2d). The electron diffraction patterns of the selected areas at the rod-like crystal region revealed a pattern of concentric rings. Five rings had d-spacings of 0.317, 0.281, 0.277, 0.262 and 0.172 nm corresponding to 102, 211, 202 and 004 indices of OH-AP.

The intermediate layers consisting of thicker mineral deposits were formed at the region between dental calculus with mineralized bacteria and denture resin. They were classified into three types. The first type consisted of densely-packed, needle-like crystallites and the contour of the interface was extremely smooth at low magnification (Fig. 3a). HREM revealed that the lattice fringes of the needle-like crystallites came in contact with the near atomic structures of resin polymer at the junction (Fig. 3b). The electron diffraction patterns of the selected areas obtained from this type of layer revealed a pattern of concentric rings. Three rings had d-spacings of 0.343, 0.281 and 0.277 nm, which corresponded to 002, 211 and 112 indices of OH-AP.

The second type of mineralized layer was thicker than the first one. Island-like structures consisting of needle-like crystallites lay scattered within the layer (Fig. 4a). HREM revealed that the lattice fringes of needle-like crystallites came in contact with the near atomic structures of resin polymer at the interface (Fig. 4b). The electron diffraction patterns of the selected areas obtained from this layer revealed a pattern of concentric rings. Four rings had d-spacings of 0.343,



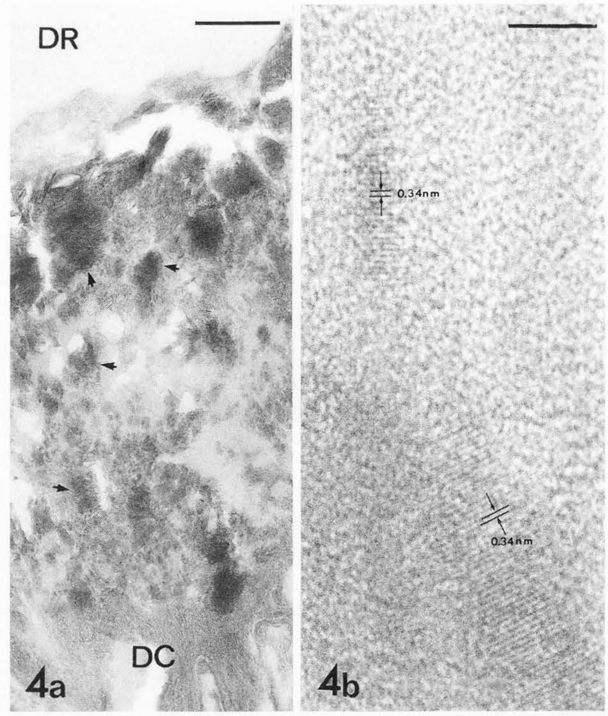


Figure 3. (**a**) A low magnification image of the intermediate layer between dental calculus (DC) and denture resin (DR). The arrows indicate a smooth contour. Bar = 0.5 μm. (**b**) A high resolution image of a fine crystallite at the interface. The lattice fringes of a fine crystallite come in contact with the granular structures of resin polymer. Bar = 5 nm.

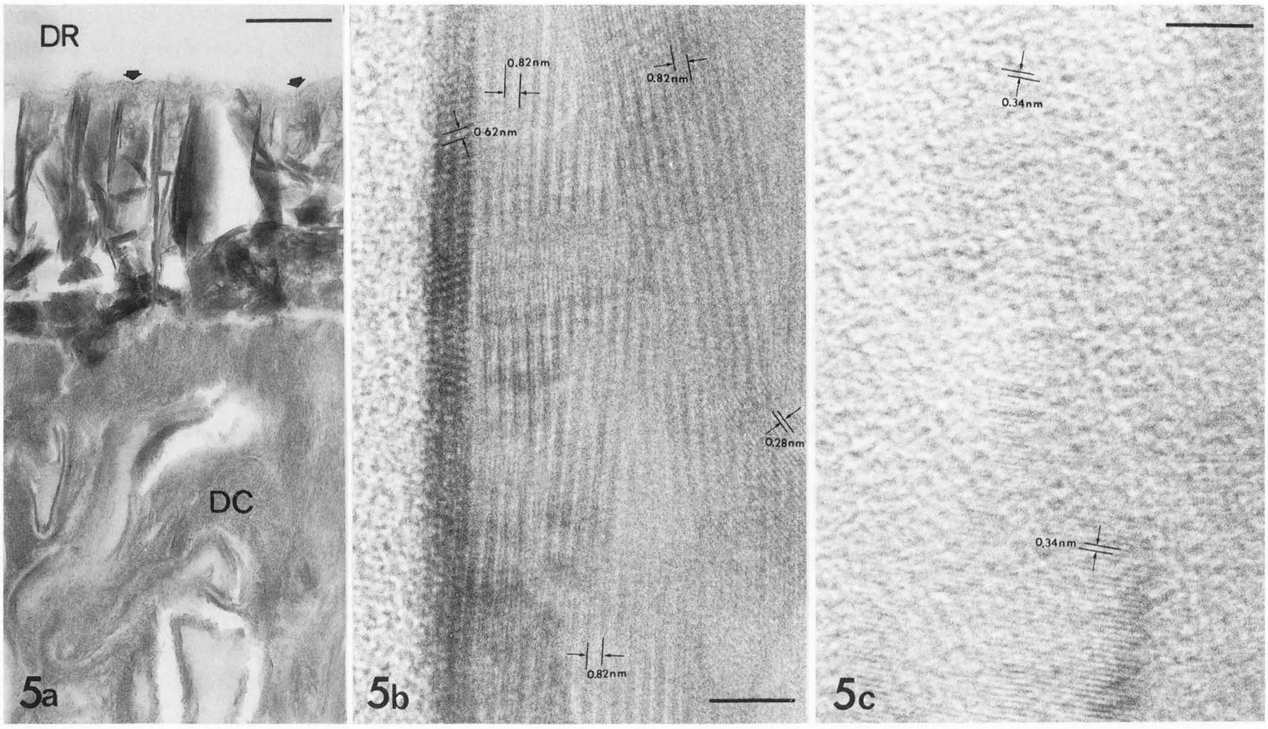
Figure 4. (**a**) A low magnification image of the intermediate layer between dental calculus (DC) and denture resin (DR). The arrows indicate island-like structures within this layer. Bar = 0.5 μm. (**b**) A high resolution image of fine crystallites at the interface. The lattice fringes of the crystallites come in contact with the granular structures of the resin polymer. Bar = 5 nm.

0.309, 0.280 and 0.277 nm corresponding to 002, 210, 211 and 112 indices of OH-AP.

The third type of mineralized layer consisted of large plate-like crystals coexisting with needle-like crystallites (Fig. 5a). HREM indicated that the plate-like crystals consisted of the aggregation of small crystallites connecting with each other at appositional levels (Fig. 5b). The lattice fringes of the crystallites came in contact with the near atomic structures at the interface (Fig. 5c). The electron diffraction patterns of the selected areas obtained from this layer revealed a pattern of concentric rings. Six rings had d-spacings of 0.820, 0.341, 0.311, 0.280, 0.276 and 0.262 nm, which corresponded to the mixture of 211, 0.0.12 and 128 indices of whitlockite and 100, 211 and 202 indices of OH-AP (Fig. 5d).

Discussion

The mineral phase of dental calculus has been extensively investigated using transmission electron microscopy and electron diffraction analysis [10, 17, 18, 20, 26, 28, 29, 30, 31, 33]. The long ribbon-like crystals were identified as OCP, the fine needle-like crystallites as OH-AP, the rhombohedral crystals as whitlockite, and the polygonal, and/or plate-like crystals, as brushite. The existence of three different types of intermediate layers between the dental calculus with mineralized bacteria and the denture resin could be demonstrated for the first time in the present study. Rod-like and plate-like crystals at a low magnification were recognized as the aggregation of small crystallites in HREM. This indicates that HREM is a very powerful means to clarify and investigate the inner structure of a crystal. Furthermore, HREM and electron diffraction analysis showed that plate-like crystals contained whitlockite crystallites. Whitlockite had already been observed in the dentinal tubule [7, 8], the fissure floor [15, 24, 25], and enamel cracks [16, 19, 21]. The appearance of whitlockite is discussed regarding the correlation with the natural repair process of hard tissue. The existence of large plate-like crystals containing whitlockite mineral might thus reasonably be thought to rapidly develop an intermediate



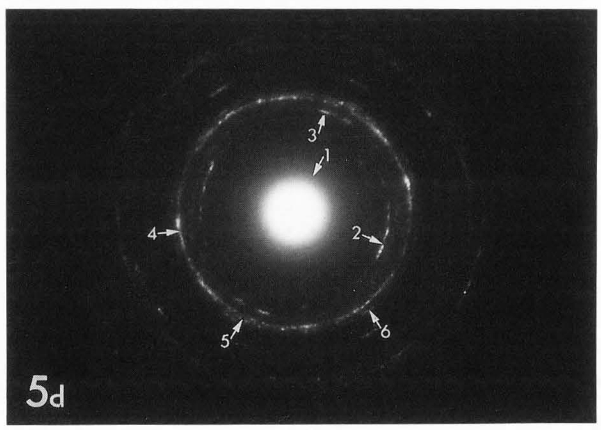


Figure 5. (**a**) A low magnification image of the intermediate layer consisting of plate-like crystals between dental calculus (DC) and denture resin (DR). The arrows indicate needle-like crystallites at the junction. Bar = 0.5 μm. (**b**) A high resolution image of a single plate-like crystal. The aggregation consists of crystallites (lattice fringes with 0.82 nm intervals) positioned in different directions at the appositional levels. Note the lattice fringes with 0.62 nm intervals characteristic of whitlockite. Bar = 5 nm. (**c**) A high resolution image of the tip of plate-like crystallite at the junction. The lattice fringes of a crystallite come in contact with the granular structures of the resin polymer. Bar = 5 nm. (**d**) Typical electron diffraction pattern of the region of large plate-like crystals in Figure 5a. D-spacings of 0.341 (arrow 2), 0.311 (arrow 3) and 0.276 (arrow 5) nm correspond to the 211, 0.0.12 and 128 spacings of whitlockite. Rings of 0.820 (arrow 1), 0.280 (arrow 4) and 0.262 (arrow 6) nm correspond to the 100, 211 and 202 indices of OH-AP.

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layer in the present situation.

Saliva is supersaturated with respect to some calcium phosphates, namely, apatite, OCP and whitlockite, and is able to support crystallite growth [11, 23]. This means that calcium phosphates do not spontaneously precipitate on the denture resin. The discrepancy between the above-mentioned phenomenon and the present findings could potentially be due to the water-adsorbable character of acrylic resin.

Generally, water was adsorbed on the polymer. Strong localized interactions develop between the water molecules and suitable polar groups of the polymer [1]. As a result of this, polymer chains are pulled apart and the water penetrates and diffuses into the polymer [3]. This phenomenon occurs at the molecular level and causes the hygroscopic expansion (swelling) of acrylic resin. In the oral cavity, the water of saliva could similarly penetrate into the acrylic resin [2]. Calcium and phosphate ions in saliva are trapped in the molecules of the acrylic resin through above-mentioned mechanism. Since the supply of these ions from saliva is sufficient, a local ion concentration gradually increases to reach supersaturation, whereas a spontaneous precipitation would occur at the surface layer of the denture. After this step, the mineral deposition is thought to progress through the additional mineralization originating from the saliva. The lattice fringes of the fine crystallites were observed in association with the near atomic structures of the resin polymer. As a single crystallite at the interface was less than 10 nm in width and ultrathin sections were 70-80 nm in thickness in the present study, the possibility of an overlap between the lattice fringes of crystallites and the near atomic structures of resin polymer is therefore undeniable. However, the contact between the lattice fringes of the crystallites and the granular structures corresponding to polymer chains was recognized at the marginal regions of the lattice fringes on the same focus plane. Therefore, at high resolution electron microscopic level, this finding proves that the mineral precipitation would progress in the spaces among the polymer chains.

It is considered important to determine how deeply crystallite formation occurs in the surface layer of the denture. As specimens were embedded in epoxy resin to make the sectioning easy and to provide ultrathin sections which are stable in HREM, it was difficult to differentiate the acrylic resin from epoxy resin in the HREM image. The mineral deposits attached to the denture side measured about 1 μm in the widest region in the ultrathin sections, with artificial spaces between calculus and denture. These findings are also supported by the fact that saliva has higher viscosity than water. The crystallite formation is therefore thought to be restricted to an extremely superficial layer of the denture resin. Furthermore, a thin layer of newly formed crystallites would be sufficient for scaffolding of the additional mineralization on the denture surface. Presently, composite resin is universally used for dental caries prevention and initial caries treatment [6]. The present findings suggest the possibility that the natural repair through the mineral deposition could be an effective means to occlude the microspace between composite resin and enamel which is caused by fracture and/or defacement of the resin.

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Discussion with Reviewers

G. Daculsi: What is the age of the prosthesis? Do you have some comments about the "maturation" of such a calculus on the resin?

Author: The patient used this denture for about 3 years. In summary, calculus maturation takes place in four steps. The first step is the penetration of the water of the saliva into the superficial layer of the resin. The second is formation of fine needle-like crystallite formation among granular structures of the resin polymer. This step is the most important and unique point in this study. As mentioned in the **Discussion**, HREM revealed that mineral precipitation would progress in the spaces between the polymer chains. There is no previous report that crystallites are penetrating a non-fluid substance. Hence, the present report is the first one observing such a situation. This phenomenon is probably due to the diffusion of the water {containing Ca and inorganic phosphate (P_i) ions} of the saliva into the denture resin. The third step is the formation of an intermediate layer which acts as scaffolding in calculus formation. The final step is the process of bacterial mineralization.

G. Daculsi: Can you explain why you found essentially HA in such calcification. Generally, we have identified other calcium phosphates than those you indicated in **Discussion**.

Author: The denture supplied for the present study had a relatively clean surface and no macroscopically visible calculus was observed. Macroscopically visible calculus on the resin surface might consist of other phases of calcium phosphates.

G. Daculsi: How do you explain that the majority of the crystals observed did not present the large lattice plane of HA (100, 0.82 nm)? All of your crystals revealed 0.34 and 0.28 nm.

Author: The most probable reason is that fine needle-like crystallites are considered to be the most likely structures to penetrate into and grow in the superficial layer (at the interface) of the resin polymer, compared with other types of crystallites observed at the gingival pocket and fissure floor.

J. Theilade: How do you know that the space designated in Figure 1a is an artifact?

Author: This figure shows the superficial layer of the resin where rod-like crystallites are formed up to about 35 μm in depth of a narrow resin crack. An aggregate consisting of rod-like crystallites in the resin crack, in connection with calculus on the resin surface, was observed in the other block. Furthermore, the triangular outline of mineral deposits seen at both sides of the space in Figure 1a resembles the aggregate closely. These findings suggest that external forces during tooth brushing, mastication or specimen preparation might produce this space.

J. Theilade: How do you know that the granular structure in Figure 2b is resin polymer? Could it be Formvar?

Author: There is a clear difference between HREM images of resin polymer and images of Formvar membrane. Formvar membrane was observed as an almost amorphous structure on the negative films and no special structures were recognized on prints.

H.J. Höhling: Does the acrylic resin surface of the denture represent a real matrix for crystal nucleation or more a template for the apposition of saliva molecules which serve as matrix for crystal nucleation?

Author: The resin surface can become wet through the absorption of the water of saliva. Ca and P_i ions in the water of saliva penetrate into the resin matrix through diffusion. Ca and P_i ions are trapped in the molecular chains of resin polymer. I think that the resin represents neither a matrix nor a template for crystal nucleation.

H.J. Höhling: Have you found in all platelets that they are composed of smaller needles or do several platelets represent the character of single crystals in which the origin of smaller needles is no more recognizable?

Author: All plate-like and rod-like crystallites were observed as polycrystalline aggregate in the present HREM.

H.J. Höhling: Are the platelets really partly whitlockite; we have observed in our analyses that the whitlockite crystallites represent more a rhombohedral appearance, not an appearance of platelets.

Author: Crystallites having a rhombohedral appearance could not be found in the intermediate layer. I have also observed rhombohedral whitlockite crystallites at the fissure floor in human enamel. As d-spacing of 0.82 nm corresponds to 100 index of OH-AP, the mineral deposit (d-spacing of 0.62 nm in Figure 5b corresponding to 006 index of whitlockite) and some of the needle-like crystallites coexisting between plate-like crystallites are thought to be whitlockite. Whitlockite crystallites in the present study would anyhow represent an immature stage concerning the crystal size.

T. Kodaka: The sample of a denture resin was from a patient of unknown age and sex, and furthermore, the length of its exposure to the oral cavity was not indicated. There were also other various unknown conditions such as whether brushing took place or not, whether such brushing was performed with or without an abrasive paste, whether plaque-removing agents for denture plates were employed or not, and whether preservation in the air or water was undertaken after the denture plate was taken off. When the denture resin is exposed to the oral cavity for a longer period, whitlockite (WH) might be precipitated besides AP; while for a short period, dicalcium phosphate dihydrate (DCPD), octacalcium phosphate (OCP), and/or AP might be deposited. Ooya [25] and Nakagawa [24] indicated that hexahedral WH crystals were not observed in the fissures of young teeth, but were present in those of old teeth. In the crevices of enamel tufts and lamellas, hexahedral WH crystals were occasionally present only in those old teeth [21].

Author: The partial denture was obtained from a woman in her sixties. She used it for about 3 years. A dentist in charge at the Department of Prosthetic Dentistry stated that this denture was maintained relatively clean and that the patient's oral hygiene was also good. However, no data are available about the use of abrasive paste and/or plaque-removing agents. As no macroscopically visible dental calculus was recognized in this case, the proper maintenance of the denture could have prevented calculus formation, even though the denture was used for a long period in oral cavity. The thin outer layer of calculus (about 120 μm, in the thickest region in the present study) consists of needle-like apatite crystallites. Although I have not yet examined the macroscopically visible thick calculus on the denture surface, the present findings might suggest that the conditions of maintaining the denture could influence the process of calculus maturation, including the morphology and chemical composition. Furthermore, the inner intermediate layer, consisting of mineral deposits, showed special unique shapes. These structures are thought to be suitable for the connection between denture resin and calculus containing mineralized bacteria.

T. Kodaka: In the **Results**, the illustrations of figures on the interface of calculus and the resin are not sufficient. I can not distinguish the calculus crystallites from the resin.

Author: Resin polymer shows granular structures in HREM image (magnification: x3,000,000 in the present figures). Fine parallel lines together with a number (unit: nm) indicate lattice striations. These granular structures can be observed even at the interface.

T. Kodaka: The author should use denture plates under as simple conditions as possible. In addition, the author should use denture plates exposed to the oral environment for short and longer periods.

Author: My professional field is operative dentistry and endodontology: caries treatment and prevention after treatment. Presently, composite resin is universally used as restorative material. Marginal microleakage and microfracture following filling are clinically important questions. It is important how to respond to these phenomena. Therefore, I chose the denture resin as a simple basic model, and investigated the mechanism of mineral deposition on the denture resin, in particular, the possible contact at the junction between dental calculus and denture resin. The fine needle-like structures would likely function as scaffolding within the resin polymer, compared with the hexahedral WH crystallites observed at the fissure floor.

T. Kodaka: Is the HREM image of denture resin a case without any exposure to oral cavity? Is there any difference between the exposed denture resin and epoxy resin for embedding in HREM?

Author: As dehydration and heat polymerization are carried out during specimen preparation, it is thought to be fundamentally similar. Polymers of denture resin are observed as complicated three-dimensional granular structures similar to those of epoxy resin. As epoxy resin penetrating at the artifact regions can be easily distinguished from the denture resin by the clear interface between two types of resins, these epoxy resin regions were never used for the present HREM figures.

T. Kodaka: In **Results** and **Discussion**, the author reported hydroxyapatite (OH-AP). Recently, however, biological apatites (AP), especially those of dental calculus, have been called carbonate apatite (see e.g., LeGeros [34]). Please comment.

Author: Carbonate, CO_3^{2-} is substituted for OH^- and/or PO_4^{3-} in carbonate apatite. However, as the existence of CO_3^{2-} could be generally identified in the infrared or Fourier transform infrared absorption spectra, in my opinion, it is impossible to evaluate the existence of carbonate apatite only from the present data using HREM and selected area electron diffraction.

T. Kodaka: Is your description "the water of saliva could similarly penetrate into the acrylic resin" based on your data? Saliva should show a higher viscosity than water. The denture resin might have occasionally been preserved in water.

Author: This property of the resin polymer is generally accepted [2]. The phenomenon is clinically observed following composite resin filling. Preservation in water maintains physico-chemical characteristics preventing the dryness of resin. In addition, bacterial mineralization within the plaque on resin surface might be inhibited due to the diffusion of Ca and P_i ions into water.

Additional Reference

[34] LeGeros RZ (1991). Calcium Phosphates in Oral Biology and Medicine. In: Monographs in Oral Biology, Vol. 15. Myers HM (ed.). Karger, Basel, Switzerland. pp. 1-201.