Rate of pellicle formation in vivo

Kjeld K. Skjørland, Morten Rykke and Torleif Sønju
Faculty of Dentistry, University of Oslo, Oslo, Norway

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The acquired enamel pellicle is thought to be the result of a selective adsorption of salivary proteins and to be involved in the protection of the enamel surfaces. The chemical composition of the 2-h acquired enamel pellicle is fairly well established. However, the rate of formation and the amino acid composition of the initially formed enamel pellicle have been little investigated. The aim of this study was therefore to examine the rate of pellicle formation and the amino acid composition of the initially formed enamel pellicle. Samples of human enamel surfaces were carried in the mouth for various periods of time (2.5 min to 10 h). Rate of pellicle formation was indicated as a function of oral exposure time and the time necessary to remove the proteinaceous film from the surfaces by argon ion sputtering. The chemical composition of the initially acquired pellicle was examined by amino acid analyses of pellicle material collected in vivo from enamel surfaces 15 min and 1 h after pumicing, respectively. The pellicle reached an initial thickness in about 2–3 min, at which level it stayed for about 30 min. The thickness of the acquired pellicle then increased to about three times the initial thickness and stayed at that level for the rest of the experimental period (10 h). Amino acid analyses of pellicle material collected after 15 min and after 1 h were different in that the amino acid profiles of the 15-min pellicle only contained traces of proline and arginine. It may be argued that the pellicle formation proceeds in two stages owing to the adsorption of protein aggregates and that the chemical compositions of the pellicles of the two stages differ. □ *Amino acid analyses; Auger electron spectroscopy analysis; pellicle formation; protein adsorption*

Morten Rykke, Department of Cariology, Faculty of Dentistry, University of Oslo, P.O. Box 1109, Blindern, N-0317 Oslo, Norway

It is generally agreed that the acquired enamel pellicle is the result of a selective adsorption of salivary proteins to the enamel surfaces and that it provides protection for the surfaces (1–4). The rate of pellicle formation may therefore be of importance for maintaining the protective functions after its removal and thus avoiding damage to the enamel surfaces. This is of special interest as agents introduced in prophylactic procedures have been shown to interfere with pellicle formation (5, 6). However, the rate of the initial pellicle formation has been little investigated, although it is generally assumed that 2 h after pellicle removal the enamel surfaces have again become more or less covered by an acquired enamel pellicle (7–10). Lenz & Mühlemann (11) examined pellicle thickness by means of scanning electron microscopy; they found a maximum thickness of about 1 μm and even found the same thickness after several days. Sönju et al. (10) found by transmission electron microscopy that 2 h after pumicing the enamel surfaces were covered by an organic film. Sönju & Rölla (9), using amino acid analysis, found that the acquired enamel pellicle increased in thickness for about 1 h and that the rate of pellicle formation then leveled off. Öste et al. (8) concluded that the final thickness of the acquired enamel pellicle was reached after 1–2 h. Kuboki et al. (7), using X-ray photoelectron spectroscopy (XPS), examined the adsorption of proteins to enamel surfaces that had been carried in the mouth. The relative mass of nitrogen indicated the presence of organic material. The samples were taken after 30, 60, 90, and 120 min and showed that the amount of proteins increased from 30 min and then reached a plateau after about 90 min. The surface morphology of this acquired enamel pellicle has been described as being predominantly globular or with a knotted surfaces structure on the basis of scanning electron microscopic examinations (12, 13).

Differences in the chemical composition of the acquired enamel pellicle after various times of formation have been investigated as well. Sönju & Rölla (9) were unable to detect differences in the amino acid composition of the acquired pellicle from 10 min to 1 h. Bennick et al. (14) found little variation in the chemical compositions between the 1-h and the 24-h pellicle, except for a gradual protein degradation. Rykke et al. (15, 16) compared the amino acid composition of the 2-h pellicle from three individuals over a 2-year period and found similar amino acid compositions and only small differences in the chemical compositions of the 24-h pellicle. They also found indications that the acquired enamel pellicle may contain dietary products when the individuals did not restrain from eating or drinking during the test periods. Al-Hashimi & Levine (2) also examined the 2-h pellicle, using gel electrophoresis and specific radiolabeling, which confirmed the selective adsorption of salivary proteins to the dental enamel.

The literature therefore provides little detailed information about the rate of pellicle formation and pellicle composition, especially concerning the initially formed pellicles.

The aim of the present study was therefore to obtain more information about the initial pellicle by following the formation from the first minutes and up to 10 h. It

also appeared to be of interest to compare the amino acid composition of the initially formed enamel pellicle with the known one of the 2-h pellicle.

Materials and methods

Enamel samples

Samples of human enamel surfaces (about 4 × 4 mm) were prepared from unerupted and surgically removed third molars, pumiced, and rinsed with distilled water. Three enamel samples were mounted in appliances fitting the buccal surfaces of the mandibular molars of three individuals, aged 40–60 years, with DMFS from 12–52 and with normal salivary flow rates. The enamel surfaces were carried in the mouth for periods of 2.5, 5, 15, 20, 30, and 45 min and 1, 2, 5, and 10 h. Eating and drinking (except for water) were not allowed during the test periods.

AES analysis

The enamel samples were submitted to Auger electron spectroscopy (AES) and depth profiling. The AES analyses were done in a fully automated fine-focus Auger microprobe (Varian Associates) by ELAB (Electronics Research Laboratory, University of Trondheim, Norway). AES was used in conjunction with argon ion sputtering to obtain depth profiles of the acquired enamel pellicles. The sequential sputtering time was 1/10th min, using an argon ion beam of 3 kV energy and 100 $\mu A/cm^2$ ion current density.

The detection of nitrogen and oxygen on the enamel samples indicated the presence of a proteinaceous material in the form of an acquired enamel pellicle. The disappearance of nitrogen, concomitantly with a sudden increase of calcium, indicated the beginning of the enamel surface.

Pellicle collection

Pellicle material for amino acid analyses was collected in vivo from the same individuals. The pellicle material was collected from the buccal surfaces of 16 through 26 without any dental restorations or carious lesions and with a firm and healthy gingiva. The buccal surfaces were pumiced and rinsed with water, and pellicle was then allowed to form for periods of 15 min or 1 h. These periods were chosen after preliminary experiments showed them to be representative for the two levels of pellicle formation as described in the Results. After the enamel surfaces had been rinsed with distilled water and air-dried, the pellicle material was collected into polyethylene tubes filled with distilled water by carefully scraping the enamel surfaces with a sharpened Pasteur pipette connected to a suction device. The samples were frozen until analyzed. Pellicle material was collected three times from each individual at each level and analyzed separately.

Amino acid analysis

The samples with pellicle material were freeze-dried, suspended in 3 ml 6N HCl, evacuated, flushed with highly purified nitrogen, and sealed before vacuum hydrolysis. Hydrolysis was performed for 24 h at 108°C. The amino acid analyses were performed in a Biotronic Auto Amino Analyzer LC 5000, using ninhydrin as detector reagent and equipped with an automatic integrator.

Results

Pellicle formation

The rate of pellicle formation of the three individuals in shown graphically in Fig. 1 as a function of the oral exposure time (the abscissa) and as the time necessary to remove the organic material from the surfaces by argon ion sputtering (the ordinate). The sputtering time is related to the amount of organic material adsorbed to the enamel surfaces. The pellicle formation was fast and seemed to proceed in two steps. An initial thickness corresponding to about 3/10th min sputtering time was reached within the first 2.5 min of pellicle formation. The sputtering time did not increase for the next 20–30 min, whereafter it increased to about 9/10th min. Only minor variations in sputtering time were found after 2, 5, and 10 h. The rate of pellicle formation of the three individuals followed the same general pattern and showed only small variations.

Chemical composition

The results of the amino acid analyses of the 15-min and 1-h pellicles as shown in Fig. 2. The results are the mean of three samples from each individual at each level (15 min and 1 h). The figure shows the different amino acids along the abscissa, and the amounts are calculated as moles per 100 mol on the ordinate.

The amino acid profiles of the 1-h pellicles were characteristic in that the acidic and neutral amino acids were the most abundant, accounting for 60% of the total. Glutamic acid and glycine were the two most abundant amino acids, comprising about 25% of the total. Only minor amounts of the sulfur-containing amino acids methionine and cysteine were detected. No amino acids characteristic of bacteria, such as diaminopimelic acid, β-alanine, or muramic acids, were detected.

Samples of the two stages of pellicle formation appeared to differ in their amino acid compositions. The 15-min pellicle contained abundant acidic and neutral amino acids as well, comprising 65% of the total. However, the 15-min pellicle contained only traces

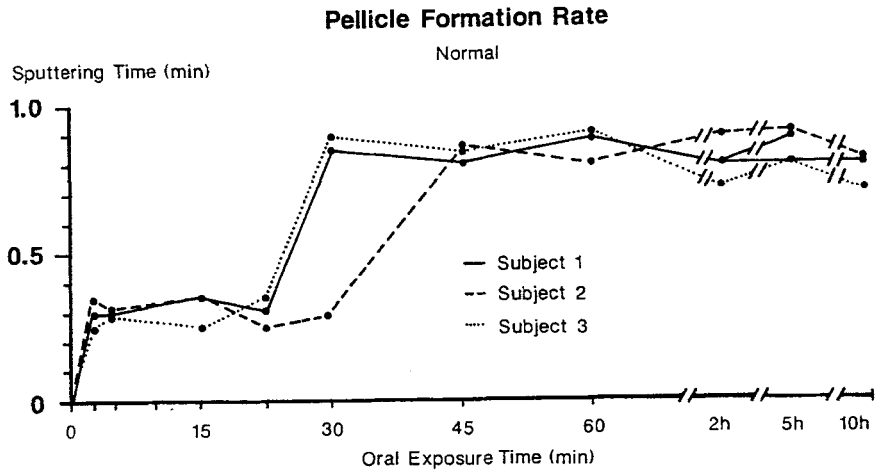

Fig. 1. Pellicle formation rate of the three individuals as a function of oral exposure time and time necessary to remove the organic material by argon ion sputtering. Sputtering time indicates pellicle thickness on the ordinate.

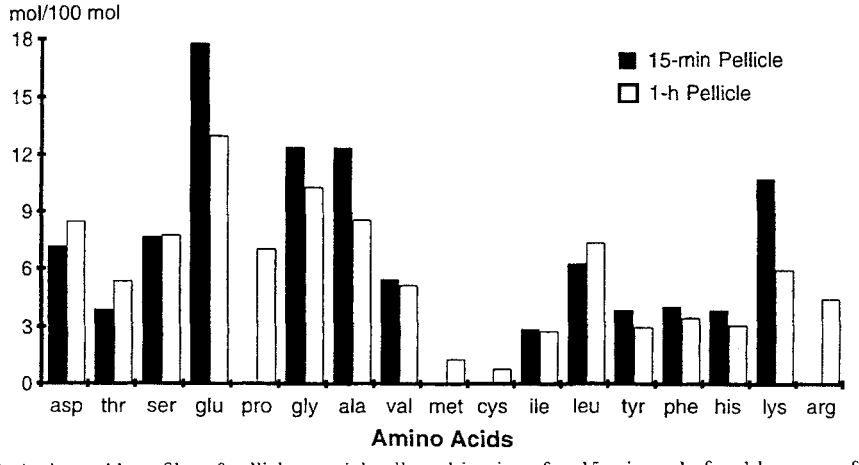

Fig. 2. Amino acid profiles of pellicle matrial collected in vivo after 15 min and after 1 h; mean of three samples from each individual at each level. Amino acids demonstrated are listed along the abscissa and amounts are shown on the ordinate as moles per 100 mol.

of proline and arginine, as compared with 7.5% proline and 5% arginine in the 1-h pellicle. The 15-min pellicle also contained significantly higher amounts of glutamic acid, alanine, and lysine.

The AES analysis and depth profiling showed the presence of carbon, sulfur, and phosphorus in the 15-min pellicle. However, sulfur decreased towards the enamel surface. In the 1-h pellicle sulfur was detected when more than half the thickness of the pellicle material was removed, as judged by the sputtering time.

Discussion

There are few investigations concerning the rate of pellicle formation in vivo, and most have been performed with too long intervals between the samples to obtain detailed information about the initially formed enamel pellicle. Kuboki et al. (7), using the XPS technique, examined the first sample after 30 min and were consequently unable to obtain information about the first minutes of pellicle formation.

In this study the sputtering time necessary to remove the organic material indicated the relative amount of organic material present. It is difficult to give a rough estimate of the pellicle thickness corresponding to sputtering time, as the pellicle material was observed in a vacuum. The thickness of the organic integument as described by this method depends on several factors, such as the packing of the organic molecules. However,

the packing of the molecules seems to have only minor influence on the sputtering yield. Therefore, the increase in sputtering time is clearly related to the adsorption of more organic material to the enamel surfaces.

The present results show that the acquired enamel pellicle may be rapidly formed again after removal. Samples prepared after 2.5 min indicated that an organic covering already was present. This is of crucial importance to avoid damage to the enamel surfaces. The present results, based on initial frequent samples, indicate that the pellicle formation may be a two-step process. One plateau was reached already after 2–3 min. The sputtering time did not increase for the next 20–30 min; however, then it increased to about three times the level of the first plateau.

This observation is interesting on the basis of the observations that the surface morphology of the 2-h acquired enamel pellicle by some authors has been described as being predominantly globular or with a knotted surface structure (12, 13). It is suggested that these surface structures may be caused by the adsorption of salivary protein aggregates in the form of globular or micelle-like structures, which recently have been demonstrated in human saliva (17, 18). Therefore, the first level of pellicle formation may well be explained by an initial adsorption of discrete proteins to the enamel surfaces, followed by the adsorption of salivary protein aggregates in the form of micelle-like structures that move more slowly towards the interfaces and hence account for the stepwise increase in the pellicle thickness. The adsorption of protein aggregates is consistent with the observation that the acquired enamel pellicle can be visualized by scanning electron microscopy at low magnifications on enamel samples carried in the mouth for 2 h (5, 19).

The initial adsorption of discrete salivary proteins to the enamel surfaces may comprise electrostatic interactions through the hydrophilic regions, leaving the more hydrophobic parts of the molecules exposed at the surface. The following adsorption of protein aggregates, or micelle-like structures, partly through electrostatic interactions to uncovered adsorption sites and partly by hydrophobic interaction to the initial protein layer, may then account for the globular surface morphology of the acquired pellicle.

The shift from side-on adsorption to end-on adsorption of the protein molecules when the surface has become completely covered by protein molecules, as proposed by Juriaanse et al. (20), may also explain the stepwise pellicle formation. However, protein adsorption is mainly described as adsorption of discrete protein molecules in monolayers and with a thickness of the proteinaceous layer consistent with the diameter of the protein molecules in solution (21, 22). The adsorption of salivary proteins in the form of micelle-like structures may therefore best explain the observed two-step phenomenon in the pellicle formation and visualization of the acquired pellicle by SEM.

The differences in amino acid compositions between the two levels of pellicle formation indicate that different proteins are adsorbed at the two different levels. It is noteworthy that only traces of proline were detected in the first level. The protein aggregates in the form of micelle-like structures have been proposed to consist mainly of amphiphilic salivary proteins, of which the proline-rich proteins (PRPs) comprise an important group (23). The presence of proline in the second level of pellicle formation may therefore indicate the presence of amphiphilic proline-rich proteins in the form of protein aggregates or micelle-like structures. Bennick et al. (14) also reported an increase in proline-rich proteins in the pellicle during the 1st h of formation. The amino acid compositions of the 1-h pellicle in the present study were also similar to the amino acid profiles of the 2-h pellicle previously reported by Rykke et al. (15). This is consistent with the observation of only minor shifts in the adsorption patterns during the rest of the experimental periods.

Pellicle material was collected from the buccal surfaces of upper teeth and from the appliances fitted on the buccal surfaces of lower molars. There is no reason to assume that this may have influenced the interpretation of the results, as it has previously been shown that the amino acid composition of the acquired enamel pellicle is the same from different teeth (9).

Carbon was detected throughout the pellicle depth. The carbon level was studied to check possible carbonization of the samples due to excess sputtering. However, the detection of carbon most probably represents glycoproteins in the pellicle material, which is consistent with the observation that glycoproteins represent a major protein component of the pellicle material (2). The detection of sulfur in the 15-min pellicle and in the deeper layers of the 1-h pellicle, together with the stepwise increase of pellicle thickness, may indicate the adsorption of protein aggregates to an initially adsorbed monolayer of discrete protein molecules. The specific adsorption of salivary proteins to dental enamel may therefore be explained in terms of adsorption of mainly amphiphilic proteins forming protein aggregates or micelle-like structures.

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