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SHORT COMMUNICATION

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Atomic force microscopy study of salivary pellicles formed on enamel and glass in vivo

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Abstract This study was performed to evaluate the use of atomic force microscopy (AFM) in examining the surface of the adsorbed layer of salivary proteins (salivary pellicle) formed in vivo on dental enamel and glass surfaces. Enamel and glass test pieces were attached to the buccal surfaces of the upper first molar teeth in two adults using removable intraoral splints. The splints were carried intraorally over periods ranging from 10 min to1 h. Using the contact mode of AFM, pellicle structures could be recognised on intraorally exposed specimens compared to nonexposed enamel and glass surfaces. The surface of the adsorbed salivary pellicle was characterised by a dense globular appearance. The diameter of the globulelike protein aggregates adsorbed onto enamel and glass varied between 80 and 200 nm and 80 and 150 nm, respectively. The structure of the adsorbed protein layer was clearly visible on glass surfaces, even though minor differences in the protein layer between glass and enamel specimens were observed. This study indicates that AFM is a powerful tool for high-resolution examination of the salivary pellicle surface structure in its native (hydrated) state. AFM avoids artefacts due to fixing, dehydration and sputter-coating which occur with scanning electron microscopic analyses.

Key words Atomic force microscopy · Salivary pellicle · Protein adsorption · Enamel · Glass surface

Introduction

Water-soluble biopolymers have a great impact on numerous areas of applied medical science, such as bioengineering, implant research, biocompatibility and bioadhesion processes. Cellular adherence, bacteria adhesion and adsorption of protein molecules at solid–liquid interfaces are largely influenced by the structure and physicochemical properties of the different surfaces involved. In particular, the formation of protein layers on artificial and biological surfaces is essential for biocompatibility and for the protection of functional interfaces in almost any living system. Within the oral cavity all natural and artificial solid surfaces exposed to saliva become coated with a layer of proteins and related biopolymers, described as an acquired salivary pellicle [1–3]. Pellicle formation is the result of salivary protein adsorption at the tooth–saliva interface [1–5]. A number of salivary components have been identified to adsorb onto the enamel surface, for example, phosphoproteins, acidic proline-rich proteins, salivary α-amylase, immunglobulins, mucins, lipids and glycolipids [1, 4–8]. The pellicle layer plays a critical role in determining the biocompatibility of any solid materials placed in contact with saliva. Ultrastructural studies of the pellicle layer on different solid surfaces could contribute to a better understanding of the pellicle formation and mechanisms involved. Many reports deal with the ultrastructural appearance of the in vivo formed pellicle on enamel surfaces using scanning electron microscopy (SEM) or transmission electron microscopy (TEM) [9–11]. This study was performed to evaluate the use of atomic force

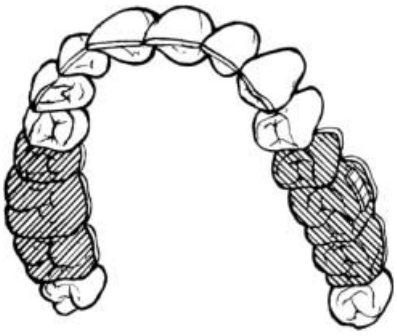


Fig. 1 Schematic drawing of the upper jaw showing the acrylic appliance (*hatched areas*) with test pieces fixed at the left buccal site of the maxillary first molar teeth

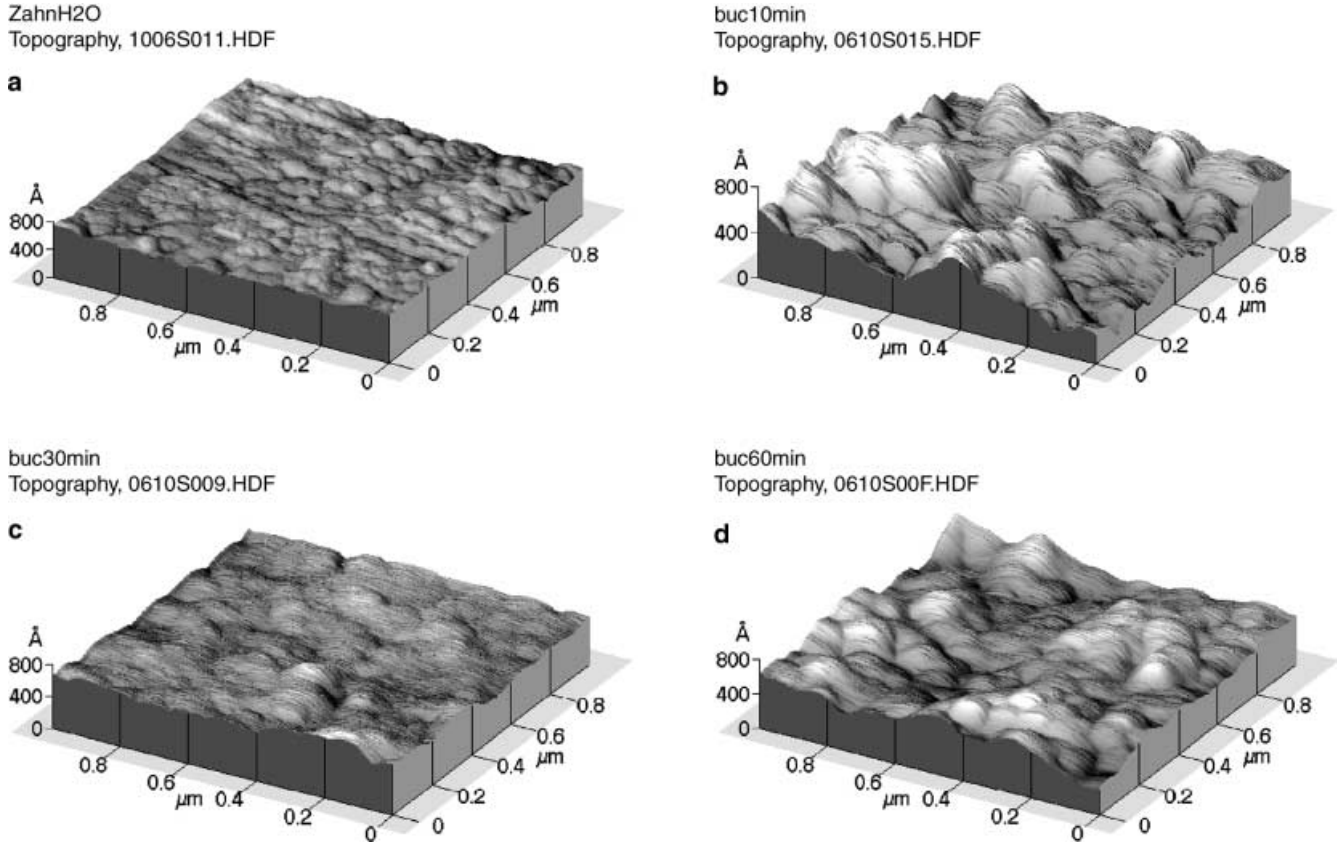
microscopy (AFM) in examining the surface of the salivary pellicle formed in vivo on dental enamel and glass surfaces.

Materials and methods

Enamel specimens with a surface area of about 2 × 2 mm were cut from labial surfaces of freshly extracted bovine incisors. The surfaces of the enamel specimens were subjected to uniform wet-grinding and polishing with abrasive paper using grit sizes down to 4.000. The specimens were disinfected using 70% alcohol. Glass specimens with a surface area of about 2 × 2 mm were cut from commercially available glass cover slices. Intraoral exposure of specimens took place by use of removable acrylic appliances (minisplints, for details see Refs. [9, 12]).

Specimens were fixed to the splints at the buccal aspect of the maxillary first molars (Fig. 1). The splints with fixed specimens were exposed to the oral environment in two subjects and carried over periods of 10, 30 and 60 min. During intraoral exposure, the consumption of food and beverages as well as any measure of oral hygiene were not allowed. Intraoral exposure of the enamel and glass specimens was repeated twice by the subjects. After intraoral exposure the specimens were removed from the acrylic splints, rinsed in distilled water and immediately mounted in the AFM sample holder. Measurements were performed using an Autoprobe CP including a 5-μm scanner (Thermo Microscopes, Sunnyvale, Calif.) operated in contact mode. Imaging was carried out using triangularly shaped silicon nitride cantilevers containing pyramidal tips (Microlever ML06F, Thermo Microscopes, Sunnyvale, Calif.). Images were taken in constant-force mode with typical scan rates and forces of 10 Hz and 17.5 nN, respectively. For reference purposes, enamel and glass specimens not exposed in the oral cavity were investigated by AFM under exactly the same conditions. Analysis of the AFM images were performed using the Park Scientific Instruments software package supplied with the AFM instrument.

Fig. 2a–d Characteristic surface pattern of polished enamel specimen. **a** Reference surface, not exposed to the oral environment. The individual enamel crystallites measuring approximately 30–90 nm. The enamel crystallites of samples exposed for **b** 10 min, **c** 30 min and **d** 60 min towards the oral environment are masked owing to salivary protein adsorption (pellicle formation)



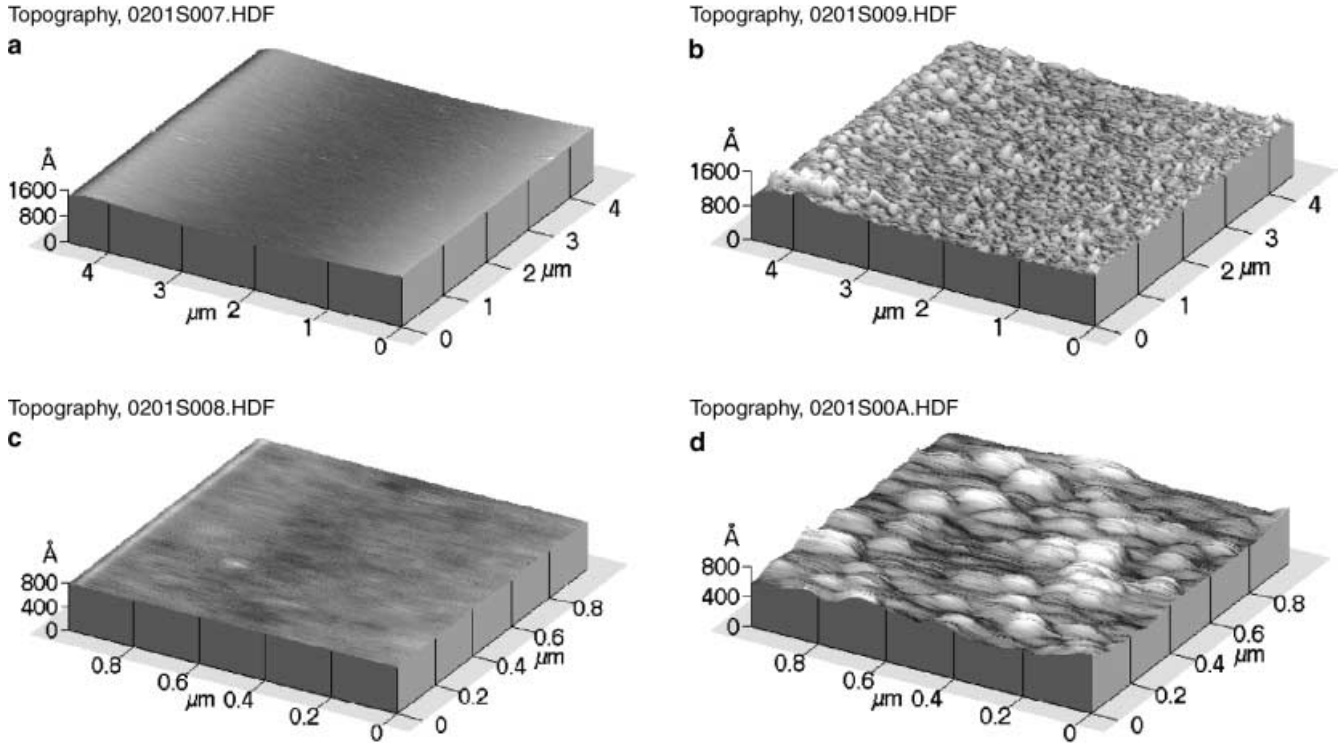


Fig. 3 The characteristic surface texture of glass specimens not exposed to the oral environment (**a**, **c**) and after exposure to the oral environment for 60 min (**b**, **d**). The formation of the salivary pellicle owing to protein adsorption is clearly discernible from the smooth reference surface

Results

The typical surface pattern of a polished enamel and glass specimen not exposed to the oral environment is shown in Figs. 2a and 3a. In the case of the enamel specimen individual crystallites measuring 30–90 nm in width could be observed by AFM. The enamel crystallites depicted in Fig. 2a appeared rounded and caused a dotted or spotted enamel surface pattern. The glass specimen shown in Fig. 3a exhibits a very smooth and structureless surface.

The intraoral exposure of both kinds of test pieces for different periods of time distinctly changed their surface appearance. The finely patterned surface structure of the reference enamel (Fig. 2a) disappeared owing to salivary protein adsorption and pellicle formation. Even after 10-min exposure of the enamel specimens to the oral cavity, the characteristic surface pattern of the enamel sample could not be detected anymore (Fig. 2b). Pellicle formation manifested itself in the AFM pictures as a tightly packed globular surface layer, presumably formed from interdicting globular protein molecules after longer exposure times (Fig. 2c, d). From the AFM pictures taken so far a rough estimation of the coil dimensions of the protein aggregates in the hydrated surface layer could be made. The average diameter of the globular-like structures varied between 80 and 200 nm (Table 1). The time-related progress of the pellicle formation is well reflected in the estimated coil dimensions. After 10 min of intraoral exposure, AFM analysis showed a variably globular structured pellicle surface with distinct globular agglomerates of adsorbed salivary proteins (Fig. 2b), whereas after intraoral exposure times of 30 and 60 min, respectively, the globular surface coating appeared denser and more homogeneous (Fig. 2c, d). These results are even more underlined by the AFM images of glass specimen presented in Fig. 3. At low magnification the surface of the reference glass specimen not exposed intraorally is smooth and homogenous (Fig. 3a). The formation of the pellicle layer on the glass surface after 60-min intraoral exposure time is well reflected in the AFM image (Fig. 3b). At higher

Table 1 Supposed pellicle structure and coil dimension of the adsorbed biopolymer estimated from atomic force microscopy

Sample	Exposure time (min)	Surface structure	Coil dimension (nm)
Enamel	0	Crystallites	–
	10	Heterogeneously globular	80–200
	30	Homogeneously globular	200
	60	Heterogeneously globular	80–180
Glass	0	Smooth	–
	60	Heterogeneously globular	80–150

magnification the change in surface structure of the glass sample is much more obvious (Fig. 3c, d) and is virtually comparable to the alteration observed for the enamel specimen depicted in Fig. 2.

Discussion

The initial stages of pellicle formation are of great interest in the field of preventive dentistry but also in relation to all surface phenomena in the oral cavity; however, the exact mechanism of pellicle formation and the many parameters involved in the adsorption process of biopolymers on intraorally exposed solid surfaces are not completely understood. Electron microscopic techniques do not enable analysis of protein layers in their native state. Artefacts resulting from sample preparation as well as dehydration of the specimens in the high vacuum of the electron microscope chamber strongly limit the validity of electron microscopy in studying the initial stages of biofilm formation on dental tissues. The environmental SEM technique can avoid such dehydration effect owing to the relatively low vacuum in the sample chamber of the electron microscope but the lateral resolution is strongly reduced. AFM allows surface analysis of wet and fully hydrated specimens without prior evaporation or special fixation procedures and a lateral resolution in the lower nanometre scale. The usefullness and applicability of AFM in studying the ultrastructure of the tooth surface have been investigated and proven by several authors in the last few years [13–15]. The results of these studies indicate that high-resolution images of dental tissues without artefacts occurring owing to dehydration are readily obtained by AFM [13, 14]. The present study used AFM to elucidate the surface ultrastructure of the native salivary pellicle formed on enamel and glass surfaces in vivo. The present AFM investigation confirms previously published SEM and TEM studies showing that the salivary pellicle layer has a globular surface structure [1, 9, 12, 16–18]. The results of the present investigation indicate that AFM gives high-resolution images of the pellicle layer adsorbed on the enamel surface. AFM, therefore, is an important tool as a source of complementary and new ultrastructural information in pellicle formation. This AFM investigation for the first time reveals evidence that the native salivary pellicle is indeed composed mainly of globular-shaped protein agglomerates which are not artefacts resulting from dehydration or agglomerates of condensed salivary proteins due to the fixing procedure. Pellicle formation is the result of salivary protein adsorption at the tooth–saliva interface [1–5]. The in vivo formation of the pellicle layer from saliva cannot be discussed without any assumptions. The first one is related to the composition of saliva and the structure of its different constituents. Human saliva contains a large number of proteins which are amphiphilic in character. Different species of salivary components have been identified: protein structures, such as phosphoproteins and acidic proline-rich proteins, functional proteins, such as salivary α-amylase and immunglobulins, and different mucins, lipids and glycolipids, which are all able to adsorb on the enamel surface. These proteins will wet and coat any natural or synthetic surfaces exposed to the oral environment, thereby forming a pellicle layer on the solid surfaces.

The outer layer of the pellicle formed on enamel and glass slabs appeared to consist mainly of a multilayer of small globules varying in diameter. It has been reported previously that most of the parotid salivary proteins appear as globular structured aggregates with a diameter of 100–200 nm [19–21], which is in good accordance with the dimensions of the globular components observed by AFM. The globules are suggested to have a hydrophobic interior and a negatively charged surface and may represent micellelike structures [19, 22]. The AFM

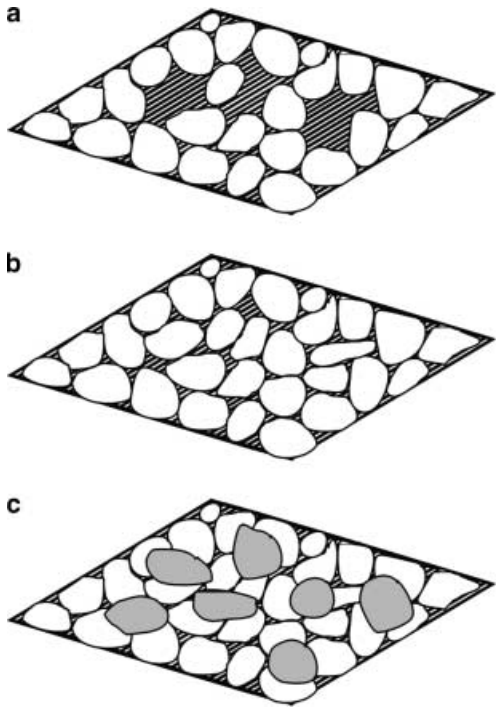


Fig. 4a–c Model for the stepwise adsorption of isolated protein agglomerates and the possible orientation of biopolymer molecules on the enamel surface. **a** Adsorption of isolated globular protein aggregates (micellelike globuli) and formation of holey pellicle surface layer. **b** Further adsorption of globular protein aggregates levels off the holes between the previously adsorbed proteins and forms a homogeneous pellicle surface layer (continuous consolidation). **c** Additional adsorption of further globular protein aggregates probably causes a more heterogeneously structural pellicle surface

micrographs indicate that these micellelike structures or protein aggregates originating from parotid saliva could be the major component of the buccally formed pellicle layer on enamel and glass. However, it is not possible to determine from the AFM pictures whether the globular protein aggregates actually exhibit micellelike characteristics after adsorption in the pellicle layer.

The pellicle surface as depicted by AFM appeared more homogeneously structured after the 30-min period of intraoral exposure compared to the 10-min period. These findings are in good accordance with previously published TEM observations and data obtained from Auger analysis of 10-min and 30-min buccally formed pellicle layers [12, 23]. These studies indicate that pellicle thickness increases rapidly within 30 min and reaches a plateau after 30–120 min [23]. Thus, the AFM figures of the 10-min pellicle layer might reflect adsorption of isolated protein agglomerates (micellelike globuli), whereas the AFM figures of the 30-min pellicle might indicate continuous consolidation and further adsorption of globular protein aggregates (Fig. 4). The further adsorption of globular protein aggregates levels off the holes between the previously adsorbed proteins and forms a homogeneous pellicle surface layer. Within 60 min of intraoral exposure, additional adsorption of further globular protein aggregates probably causes a more heterogeneously structural pellicle surface, which corroborates with recently published TEM findings [12].

In addition to enamel specimens, glass surfaces were used in the present study. The smooth and structureless surfaces of the clean glass specimens enables us to verfiy the structure and the formation of the adsorbed salivary protein layer. Indeed, only minor differences were observed in the present AFM investigation concerning the surface structure of the pellicle layer on glass compared to enamel test pieces. This observation is in good accordance with a TEM investigation showing that the pellicle layer is regularly present on various dental restorative materials and does not reveal marked ultrastructural differences [9].

Conclusions

The results of the present study indicate that AFM offers a novel way to explore the ultramorphology of the in vivo formed pellicle layer in its native state, i.e. under natural, hydrated conditions. Thus, AFM will be a useful tool for studying the initial stages of salivary pellicle formation on various biomaterials as well as for evaluation of pellicle alterations caused during acidic attacks [24]. The results of this AFM investigation provide further evidence that the in vivo pellicle formation in the oral cavity is mainly caused by adsorption of protein agglomerates/aggregates [9, 12, 19–22] rather than by adsorption of individual salivary biopolymers.

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