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ORIGINAL ARTICLE

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Transmission electron microscopy comparison of methods for collecting in situ formed enamel pellicle

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Abstract The in vivo formed salivary pellicle is composed of an outer globular and a densely structured basal layer. This study developed a method for selective recovering of these pellicle layers from the enamel surface. Two-hour in situ pellicles were formed by intraoral exposure of enamel specimens in two adults. Pellicle-covered enamel specimens were treated either mechanically (scraping with scaler, curette or razor blade, or rubbing with a sponge) or chemically (phosphate buffer, NaCl, NaOCl, $CaCl_2$, NaSCN, urea, tetrahydrofurane, guanidine, SDS, HCl, or EDTA with or without additional ultrasonication). Specimens were processed for transmission electron microscopic analysis to detect pellicle residues remaining on the enamel surface after the different treatments. Most of the chemical treatments caused partial, incomplete removal of the globular layer. Complete removal of the globular layer without disruption of the basal layer was obtained by sponge rubbing or by $CaCl_2$ combined with ultrasonication, whereas scraping caused partial disruption of the basal layer. Removal of the basal layer was observed after treatment with HCl, EDTA, or NaOCl combined with ultrasonication. Electrophoretical analysis of recovered pellicle fractions indicate that combination of sponge-rubbing followed by EDTA treatment can be recommended for stepwise removal of the globular and basal pellicle layers.

Keywords Salivary pellicle · Transmission electron microscopy · Pellicle collection

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Introduction

The acquired salivary pellicle is a proteinaceous film covering the tooth surface. The pellicle is formed by adsorption of salivary proteins onto the enamel surface [20]. Ultrastructural studies indicate that a detectable pellicle layer is adsorbed onto enamel slabs within 1 min after exposure to the oral environment [11]. Pellicle formation reaches an equilibrium between adsorption and desorption of salivary proteins within 90–120 min [18, 28, 29]. After 2-h formation time the salivary pellicle is composed of an outer layer of globular appearance and an inner densely structured basal layer [10, 11, 22, 26]. The pellicle determines all interfacial events taking place between the enamel surface and the oral environment and provides protection of the enamel against acidic attacks [12, 20]. Concerning this background, it is of great interest to understand in detail the composition and three-dimensional arrangement of proteins in the pellicle layer.

Many chemical methods and mechanical techniques have been described for removing the acquired salivary pellicle layer from the enamel surface for biochemical analyses. Upon etching with hydrochloric acid or demineralization with EDTA the pellicle layer floats off from the enamel surface and can be readily collected for subsequent analysis [3, 19, 23, 24]. Other chemical agents used for solubilization and detachment of the pellicle form the enamel surface are 2 M calcium chloride solution [8], sodium phosphate buffer [16] and sodium hypochlorite [13]. The adsorbed pellicle layer may be also mobilized from enamel specimens by ultra sonication [31]. The first method to remove and collect the pellicle layer mechanically from the tooth surfaces in vivo was introduced by Sönju and Rölla [29]. Pellicle collection was performed by scraping the tooth surfaces with a scal-er [29, 30]. More recently, chemomechanical methods have been developed for removal of the pellicle in vivo. Enamel surfaces are rubbed with sponges wetted with 2% sodium dodecyl sulfate (SDS) or swabbed with membrane filters soaked in 0.5 M sodium bicarbonate to collect the in vivo formed pellicle layer [6, 7, 38]. However, no study has yet ex-

amined the extent to which these various methods dissolve and remove the salivary pellicle layer, or whether there remain pellicle residues on the enamel after use of the different treatments. In addition, until now no method has been developed for selective recovering of the different fractions of the enamel pellicle for separate biochemical analysis of proteins in various layers of the pellicle.

The first purpose of the present study was to compare several methods for collecting pellicle material from the enamel surface with regard to their effectiveness in completely removing the in vivo pellicle layer. The extent of removal of the pellicle from the enamel surface due to different pellicle collecting methods was evaluated by transmission electron microscopy (TEM). The second aim of this study was to develop a method for stepwise, selective removal and separate biochemical analysis of the outer globular structured pellicle layer and the basal, densely structured pellicle layer.

Materials and methods

In situ pellicle formation

Enamel specimens with a surface area of approx. 2×2 mm were cut from the labial surfaces of freshly extracted bovine incisors. Specimens surfaces were subjected to wet-grinding and polishing with abrasive paper using grit sizes down to "4.000." Two healthy volunteers participated in this study. Individual intraoral appliances for mounting enamel specimens were fabricated for the volunteers in the form of acrylic minisplints covering the crowns of the maxillary premolars and the first and second molars [10, 11]. Enamel specimens were attached to the splints with a small amount of polyvinylsiloxane in the area of the buccal and palatal surfaces of the first maxillary molars. The splints with mounted test pieces were carried by the volunteers for a period of 2 h to form in situ pellicle layers. During intraoral exposure of enamel test pieces the participants refrained from any consumption of food or beverages. The study design was approved by the Ethics Committee of the University of Kiel, and informed written consent was obtained from the participants.

Removal of the pellicle

After intraoral exposure enamel specimens were rinsed with distilled water and subjected to the different chemical and mechanical treatments listed in Table 1 to remove the pellicle layer from the enamel surface. In the case of chemical treatment enamel specimens were exposed to 2 ml of the various agents without agitation for the time periods noted in Table 1. In some experimental trials chemical treatment was combined with 30-min ultrasonication (Sonorex, Bandelin, Germany). Scraping of the pellicle layer was performed by thorough treatment of the enamel surface with either a scaler, curette, or razor blade in two different directions. During scraping the instruments were moved under manual pressure over the pellicle coated surface. However, damage of the enamel surface was avoided during the mechanical instrumentation. Pellicle removal by sponge-rubbing took place with two plastic foam sponges (pellets) that were wetted with 5 µl water or 2% SDS solution. This procedure was followed by thorough rubbing of each enamel surface with two dry sponges.

Transmission electron microscopy

After the various treatments specimens were rinsed in distilled water and dropped into a 3% glutaraldehyde fixative for 2 h. Specimens were postfixed in 2% osmium tetroxide for 2 h, dehydrated in ethanol and embedded in Araldite M (Merck, Darmstadt, Germany). After decalcification in 4% EDTA (pH 7.2) and reembedding, ultrathin sections were cut on an Ultracut E ultramicrotome (Reichert, Benzheim Germany) equipped with a diamond knife. Ultrathin sections were mounted on Pioloform-F (Wacker-Chemie, Munich, Germany) coated copper grids, contrasted with uranyl acetate and lead citrate, and examined in a TEM 201 transmission electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV. Representative micrographs were obtained at a magnification of ×30,000.

At least two individual enamel specimens covered by either buccally or palatally in situ formed pellicles from each of the two volunteers were treated according to the methods described in Table 1 and investigated by TEM. For control purposes enamel specimens covered by buccal and palatal 2-h in situ pellicles without subsequent chemical or mechanical treatment as well as enamel specimens not exposed to the oral environment were examined by TEM.

Biochemical analysis

Enamel specimens with a surface area of 5×5 mm were prepared as described above. Four of these specimens were mounted at the acryl splints in the area of the buccal surfaces of the upper second premolars and first molars. Splints were carried intraorally for 2 h. Intraoral exposures were repeated five times by the volunteers to obtain enough pellicle-covered enamel slabs for subsequent biochemical analyses. Bottom and lateral sites of the enamel slabs were coated by nail varnish leaving the 5×5 mm pellicle-covered enamel surface uncoated. Enamel specimens were either rubbed with plastic foam sponges soaked with 2% SDS or treated by 30-min ultrasonication in 2 M $CaCl_2$ solution to remove the outer pellicle layer. Pellicle proteins were recovered from the sponges as described by Carlen et al. [6, 7]. After these treatments the enamel specimens were exposed in EDTA (0.4%) for 60 min to dissolve the residual basal pellicle layer. The dissolved pellicle proteins were lyophilized. For control purpose enamel specimens not exposed to the oral environment were also treated by EDTA.

For electrophoretical analysis the lyophilized pellicle proteins were dissolved in sample buffer (63 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH=6.8) to a final concentration of 2 mg/ml sample buffer. After centrifugation at 4,000 rpm electrophoresis was performed on SDS polyacrylamide gel electrophoresis slab gels, using 4% (w/v) polyacrylamide for the running gel and 10% for the stacking gel [1]. Separation of samples was carried out in the absence of 2-mercaptoethanol in the sample buffer and with delayed reduction. After separation of the different proteins by electrophoresis individual bands were visualized by silver staining according to the procedure described by Heukeshoven and Dernick [17]. Standards to determine molecular weights of unknown proteins were obtained from Bio-Rad (Munich, Germany). The standards were diluted 1:200 in sample buffer. After heating at 95°C for 5 min and quenching on ice 5 µl/well of the standards was loaded on the gels. Determination of molecular weights of unknown proteins was performed using the relative mobility (=distance migrated by protein/distance migrated by marker protein).

Results

Transmission electron microscopy

TEM findings are summarized in Table 1 and illustrated in Fig. 1. The pellicles formed within 2 h on buccally mounted enamel specimens were characterized by an electron-dense 10- 20-nm thick basal layer and a second, globular structured layer with a thickness that varied be-

Table 1 Treatment of the in situ formed pellicle layer and ultrastructural findings (appearance of the residual pellicle layer)

Treatment mode and time	Ultrastructural appearance of the buccally formed 2-h pellicle	Ultrastructural appearance of the palatally formed 2-h pellicle
No treatment (control samples)	Electron dense, 10–20 nm thick basal layer covered by a 100–300 nm thick globular layer	Electron dense, 10–20 nm thick basal layer covered by a 20–50 nm thick granular layer
0.6 M hydrochloric acid, 40–180 s	Complete removal of the pellicle	Complete removal of the pellicle
0.4% EDTA (pH 7.4), 20 min	No alteration of the pellicle	No alteration of the pellicle
0.4% EDTA (pH7.4), 40 min	Pellicle residues	Pellicle residues
0.4% EDTA (pH 7.4), 60 min	Complete removal of the pellicle	Complete removal of the pellicle
Phosphate buffer solution (pH 7.4), 24 h	Partial removal of the globular layer	Partial removal of the granular layer
15% sodium chloride, 60 min	Partial removal of the globular layer	Partial removal of the granular layer
2 M calcium chloride, 60–90 min	Partial removal of the globular layer	Partial removal of the granular layer
1 M sodium thiocyanate, 20–60 min	Partial removal of the globular layer after 60 min	Partial removal of the granular layer
2% urea, 20–60 min	Partial removal of the globular layer after 60 min	Partial removal of the granular layer
5% tetrahydrofurane, 20–60 min	Partial removal of the globular layer after 60 min	Partial removal of the granular layer
6 M guanidine hydrochloride, 24 h	Nearly complete removal of the globular layer, no alteration of the basal layer	Partial removal of the granular layer, no alteration of the basal layer
2% sodium dodecyl sulfate, 60 min	Partial removal of the globular layer, no alteration of the basal layer	Partial removal of the granular layer
Scraping with scaler/curette	Removal of the globular layer, partial disruption of the basal layer	Removal of the granular layer, intact basal layer
Scraping with razor blade	Nearly complete removal of the globular layer, partial disruption of the basal layer	Partial removal of the granular layer, intact basal layer
Rubbing with a plastic foam sponge (Pele Tim; Voco, Cuxhaven, Germany) containing 5 µl water	Removal of the globular layer	Removal of the granular layer
Rubbing with a plastic foam sponge (Pele Tim; Voco, Cuxhaven, Germany) containing 5 µl 2% sodium dodecyl sulfate	Removal of the globular layer	Removal of the granular layer
Water, 30-min ultrasonication	Removal of the globular layer, intact basal layer	Partial removal of the granular layer, intact basal layer
6 M guanidine-hydrochloride, 30-min ultrasonication	Residues of the basal layer	Residues of the basal layer
2 M calcium chloride, 30-min ultrasonication	Removal of the globular layer, intact basal layer	Removal of the granular layer, intact basal layer
15% sodium chloride, 30-min ultrasonication	Removal of the globular layer, partial disruption of the basal layer	Removal of the granular layer, intact basal layer
3% sodium hypochlorite, 30-min ultrasonication	Complete removal of the pellicle	Complete removal of the pellicle

tween 100 and 300 nm (Fig. 1). The 2-h pellicle layer formed on palatally exposed enamel slabs manifested itself in TEM micrographs as an electron-dense 10- to 20-nm thick basal pellicle layer and a second, less dense granular structured layer of varying thickness (20–50 nm; Fig. 1).

Most of the chemical treatments, such as exposure of the pellicle covered specimens to phosphate buffer solution, sodium chloride, calcium chloride, urea, tetrahydrofurane, sodium thiocyanate, and sodium dodecyl sulfate, caused partial (incomplete) removal of the outer globular or granular pellicle layer (Fig. 1, Table 1). Nearly complete removal of the globular layer was observed after treatment with guanidine-HCl. Depending on the chemical agents used, additional ultrasonication increased the effect of pellicle detachment and caused complete removal of the outer pellicle layer (in the case of calcium chloride) and partial disruption of the basal layer (in the case of sodium chloride; Fig. 1, Table 1). Scraping the pellicle-covered enamel specimens with a scaler or curette caused removal of the globular layer and partial disruption of the basal layer. However, rubbing the pellicle-covered surface with sponges resulted in complete removal of the globular or granular pellicle layer and maintenance of the basal layer (Fig. 1, Table 1). Complete removal of the pellicle layer from the enamel surface without any detectable residues left in place was observed after treatment with hydrochloric acid for 40 s, after 30-min sodium hypochlorite treatment combined with ultrasonication, or after 60-min exposure to EDTA (Table 1). The observed pattern of pellicle mobilization and dissolution due to the different treatments was found to be consistent between and within the two volunteers participating in this study. On control enamel samples not exposed to the oral environment no organic surface coatings were detected by TEM analysis.

Biochemical analysis

SDS polyacrylamide gel electrophoregrams for the selective recovering of the different fractions of the pellicle from the enamel surface are shown in Fig. 2. The pellicle samples collected by either sponge-rubbing or ultrasoni-

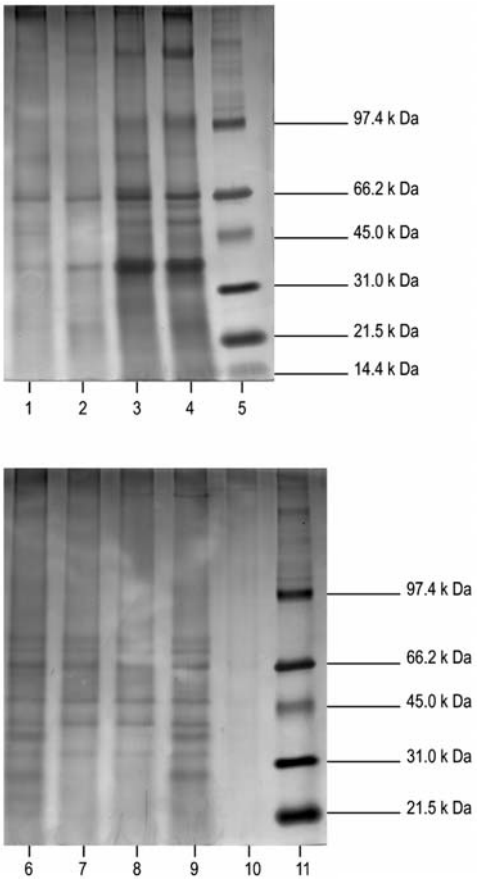


Fig. 2 SDS polyacrylamide gel electrophoresis of different pellicle fractions separated electrophoretically and stained with silver. *Lanes 1, 2* Pellicle proteins (from volunteers 1 and 2, respectively) collected by rubbing of the enamel surface with sponges soaked with 2% SDS. *Lanes 3, 4* Pellicle proteins (from volunteers 1 and 2, respectively) collected by 30-min ultrasonication of pellicle-covered enamel samples in 2 M $CaCl_2$ solution. *Lanes 6–9* Pellicle proteins recovered by subsequent 60-min EDTA treatment of the sponge rubbed (corresponding samples to *lanes 1, 2*) or ultrasonically treated specimens (corresponding samples to *lanes 3, 4*). *Lane 10* Enamel without pellicle (control) exposed to 60-min EDTA treatment. No protein bands are visible. *Lanes 5, 11* show protein bands of molecular weight standards (97.4 kDa=phosphorylase b, 66.2 kDa=bovine serum albumin, 45.0 kDa=ovalbumin, 31.1 kDa= carbonic anhydrase, 21.5 kDa=soybean trypsen inhibitor, 14.4 kDa= lysozyme)

cation in $CaCl_2$ solution were characterized by ten distinct protein bands with molecular weights of approx. 25, 31, 38, 45, 54, 64, 66, 80, 99, and 122 kDa. The pellicle fraction mobilized by subsequent EDTA treatment revealed eight distinct protein bands with molecular weights of approx. 25, 31, 35, 38, 45, 66, 70, and 75 kDa. After EDTA treatment of control specimens without pellicle layer no protein bands were identified by electrophoresis (Fig. 2).

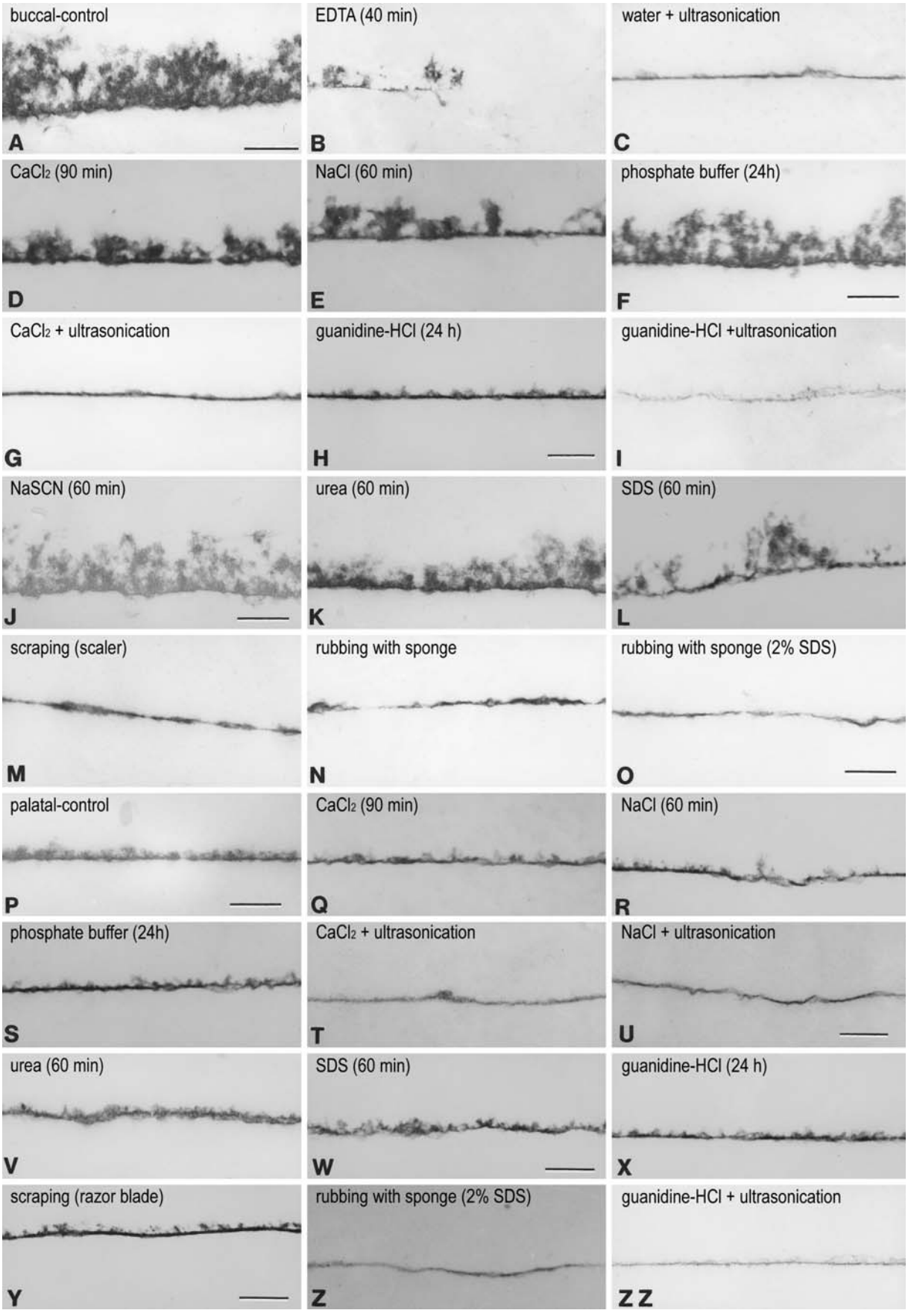
Discussion

Several methods have been described for collection and recovery of the acquired salivary pellicle layer from the enamel surface. However, until now no evidence has been provided whether these methods indeed guarantee complete removal of the pellicle layer from the enamel surface and thus allow quantitative detachment and collection of the in vivo or in situ formed salivary pellicle. Ideally pretreatment for removal and recovery of the pellicle layer should leave behind a pellicle-free enamel surface.

The present study used TEM to detect the pellicle residues remaining on the enamel surface after use of different methods for pellicle collection, since this type of analysis allows the identification of residual pellicle structures measuring only a few nanometers in thickness. Concerning the present TEM findings it appears very doubtful to completely and quantitatively remove the in situ formed pellicle layer from the enamel surface by other methods than use of chelating (EDTA) or acidic (HCl) agents, or sodium hypochlorite treatment combined with ultrasonication.

Recently published studies concerning the protective potential of the pellicle layer against acid induced erosion reveal that even after treatment with 1% citric acid for several minutes pellicle residues were found at the enamel surface [13, 14, 15]. However, as shown in the present investigation, the pellicle does not protect the enamel surface against 40-s exposure to 0.6 M hydrochloric acid. In contrast, even after 40-min exposure to EDTA (0.4%, pH 7.4) pellicle residues were detected at the enamel surface by TEM, clearly demonstrating a certain resistance of the in situ formed pellicle layer against the calcium chelating agent. The fact that no changes in the

Fig. 1 TEM micrographs of buccally (**A**–**O**) and palatally (**P**–**ZZ**) formed 2-h pellicle layers after the various treatment regimes. **A** No treatment (control sample), buccal pellicle, electron dense basal layer and outer globular layer. **P** No treatment (control sample), Palatal pellicle, electron dense basal layer and outer granular layer. **B** 0.4% EDTA (pH 7.4), 40 min, pellicle residues; **C** 30-min ultrasonication in water, pellicle residues; **D** 2 M calcium chloride, 90 min, pellicle residues; **E** 15% sodium chloride, 60 min, pellicle residues; **F** phosphate buffer (pH 7.4), 24 h, pellicle residues; **G** 30-min ultrasonication in 2 M calcium chloride, pellicle residues; **H** 6 M guanidine-HCl, 24 h, pellicle residues; **I** 30-min ultrasonication in 6 M guanidine-HCl, pellicle residues; **J** 1 M sodium thiocyanate, 60 min, pellicle residues; **K** 2% urea, 60 min, pellicle residues; **L** 2% sodium dodecyl sulfate, 60 min, pellicle residues; **M** scraping with scaler, pellicle residues; **N** rubbing with sponge containing water, pellicle residues; **O** rubbing with sponge containing 2% SDS, pellicle residues; **Q** 2 M calcium chloride, 90 min, pellicle residues; **R** 15% sodium chloride, 60 min, pellicle residues; **S** phosphate buffer (pH 7.4), 24 h, pellicle residues; **T** 30-min ultrasonication in 2 M calcium chloride, pellicle residues; **U** 30-min ultrasonication in 15% sodium chloride, pellicle residues; **V** 2% urea, 60 min, pellicle residues; **W** 2% sodium dodecyl sulfate, 60 min, pellicle residues; **X** 6 M guanidine-HCl for 24 h, pellicle residues; **Y** scraping with razor blade, pellicle residues; **Z** rubbing with sponge containing 2% SDS, pellicle residues; **ZZ** 30-min ultrasonication in 6 M guanidine-HCl, pellicle residues. Original magnification: ×30,000; *bar* 200 nm


buccal-control
A
EDTA (40 min)
B
water + ultrasonication
C
CaCl2 (90 min)
D
NaCl (60 min)
E
phosphate buffer (24h)
F
CaCl2 + ultrasonication
G
guanidine-HCl (24 h)
H
guanidine-HCl +ultrasonication
I
NaSCN (60 min)
J
urea (60 min)
K
SDS (60 min)
L
scraping (scaler)
M
rubbing with sponge
N
rubbing with sponge (2% SDS)
O
palatal-control
P
CaCl2 (90 min)
Q
NaCl (60 min)
R
phosphate buffer (24h)
S
CaCl2 + ultrasonication
T
NaCl + ultrasonication
U
urea (60 min)
V
SDS (60 min)
W
guanidine-HCl (24 h)
X
scraping (razor blade)
Y
rubbing with sponge (2% SDS)
Z
guanidine-HCl + ultrasonication
ZZ

ultrastructural appearance of the in situ formed pellicle after 20-min exposure to EDTA were recorded indicates that calcium bridging does not mainly contribute to the arrangement and stabilization of proteins and protein aggregates immobilized in the pellicle.

It is remarkable that with the exception of guanidine-HCl, which nearly completely removed the outer globular or granular pellicle layer, all the chemical treatments investigated in the present study caused only partial removal of the outer pellicle layer. However, combination of chemical treatments with 30-min ultrasonication strongly increased the extent of removed pellicle material. Thus ultrasonication can be considered as an important supportive method for recovering and collection of the in situ formed salivary pellicle. According to the present findings, buffer or salt solutions by themselves without additional ultrasonication do not seem to be appropriate for defined removal of the in situ formed pellicle.

SDS has been reported to effectively desorb proteins from both hydrophilic and hydrophobic surfaces [4, 33, 36, 37]. Based on these findings it has been proposed that salivary proteins bind the dodecyl sulfate molecules, thereby preventing deposition of the proteins due to an increase in the electrostatic repulsion forces between the surfaces and the proteins [4]. However, the present TEM results did not indicate any ultrastructural evidence that SDS is able to completely displace and desorb the 2-h pellicle adsorbed to the enamel surface under in situ conditions. In addition, also the other chemical agents, sodium thiocyanate, tetrahydrofurane, and urea which, as with SDS, are designed for dissociation of noncovalent (particularly hydrophobic) bonds and self-assembled (micellelike) structures [35] did not cause any substantial desorption of the in situ formed pellicle. These TEM findings reveal that hydrophobic interactions apparently do not mainly contribute to pellicle formation in situ.

Scraping of the enamel surface with a scaler or curette has been widely used over the past 30 years for collecting the in vivo formed salivary pellicle and subsequent biochemical analysis [2, 9, 27, 29, 30]. Although the pellicle covered enamel surfaces were thoroughly scraped in two directions in the present study, it was not possible to remove completely the entire pellicle layer. Thus scraping the enamel should be considered as an appropriate and effective method for collection of the gross amount of the in vivo formed pellicle layer, especially in intraoral areas characterized by formation of comparatively thick, globular structured pellicles. However, scraping does not guarantee recovery of the basal pellicle layer, as clearly seen in the present TEM micrographs. Similar TEM findings were recorded in the present study for the mechanical collection of the in situ pellicle layer by rubbing the enamel surface with plastic foam sponges. This method proved to be very effective for selective removal of the outer globular or granular pellicle layers, thereby preserving the electron dense basal pellicle on the enamel surface. These TEM observations are in good accordance with a previously performed AFM analysis of sponge-treated surfaces, indicating that consecutive rubbing of the surface with SDS-containing sponges does not reveal complete removal of the adsorbed protein layer [25]. Interestingly, in the present investigation wetting of the sponges with 5 µl SDS instead of water did not increase removal of the in situ formed pellicle layer. This finding clearly indicate that the mechanical action is of decisive importance, rather than the addition of chemical agents for collection of the pellicle by sponge rubbing.

It has been demonstrated previously that the overall protein composition of the 2-h in vivo formed salivary pellicles displays characteristics typical of the salivary composition and protein concentration prevailing in the area of the mouth where the pellicles are formed [6]. These findings indicate that the locally available salivary biopolymers are of importance for the pattern and extent of pellicle formation [10, 11]. Therefore in the present study pellicles from two intraoral sites which are characterized by strictly differing patterns of in situ pellicle formation [6, 10, 11] were treated by the various methods for pellicle collection. However, TEM results indicate that the general behavior of the buccally and palatally formed layers upon chemical and mechanical treatment for pellicle removal did not differ principally.

It has been suggested previously that the pellicle consists of several protein fractions adsorbed with different binding strength [32, 33, 34]. The tightly adsorbed basal layer is covered by a loosely connected outer layer [10, 11]. As can be seen in the TEM micrographs, the dense, loosely arranged outer layer of the in situ formed 2-h pellicle was much more readily dissolved in the various chemical agents than the basal parts of the pellicle.

Thus the present study provides further evidence that the basal pellicle layers reveal a comparatively higher resistance to mechanically induced abrasion or chemically induced dissolution than the outer globular layer. Therefore the basal pellicle layer might be considered the most important fraction of the pellicle regarding protection of the enamel surface [12, 13, 14, 15]. Busscher et al. [5] have proposed the hypothesis that detachment of an intraoral biofilm due to shearing forces occurs through cohesive failure in the adsorbed pellicle layer. The present TEM results for the first time confirm this hypothesis. Indeed, detachment of the in situ formed pellicle due to mechanical forces caused by rubbing the enamel surface with a sponge or by scraping the tooth surface took place at the borderline between the outer loosely arranged and the inner more densely structured basal pellicle layer.

The preliminary biochemical data presented in this study demonstrate that pretreatment by either sponge-rubbing or ultrasonication in calcium chloride solution and subsequent EDTA treatment of the pellicle layer is a suitable method for stepwise collection and separate analysis of different pellicle fractions. The number of eight to ten protein bands detected by silver staining in the various pellicle fractions is in accordance to previously reported data on the number of proteins found in the initially formed salivary pellicle [31]. The molecular weights of the protein bands detected by electrophoretical

separation in the present study correspond to molecular weights of known proteins identified in the in vivo pellicle, for example, proline-rich proteins (5–30 kDa), carbonic anhydrase (42 kDa), α-amylase (54–57 kDa), sIgA (60 kDa), albumin (69 kDa), and lactoferrin (80 kDa) [6, 21, 39]. Further studies are in progress to analyze the biochemical composition of different fractions of the pellicle at different intraoral sites.

Conclusions

Within the limitations of this ultrastructural study, the present TEM findings demonstrate that the outcome of efforts to analyze the composition of the pellicle depends crucially on the method used for collection of pellicle material. Concerning the aim of selective analysis of different fractions of the pellicle layer, stepwise removal and collection of pellicle proteins is essential. The TEM findings indicate that combining either the use of sponges for rubbing the enamel surface or 2 M $CaCl_2$ solution in combination with 30-min ultrasonication followed by 60-min EDTA treatment could be an effective way for selective removal of the outer globular and subsequently the inner basal pellicle layers.

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