Clin Oral Invest (2007) 11:211–216
DOI 10.1007/s00784-007-0112-5

ORIGINAL ARTICLE

Non-destructive visualisation of protective proteins in the in situ pellicle

Daniela Deimling · Christian Hannig · Wiebke Hoth-Hannig · Philipp Schmitz · Jürgen Schulte-Mönting · Matthias Hannig

Received: 11 July 2006 / Accepted: 27 February 2007 / Published online: 15 March 2007

Abstract Several salivary anti-microbial and buffering components are part of the acquired in vivo pellicle. The purpose of the present in situ study was to visualise these proteins within the in situ formed pellicle and to investigate their distribution with respect to pellicle formation time and intra-oral localisation. Bovine enamel slabs were fixed on individual splints. They were carried by 6 subjects buccally and palatally in the region of the upper first molar teeth over 30 and 120 min, respectively, for in situ pellicle formation. After intra-oral exposure, enamel specimens were processed for transmission electron microscopy. Secretory immunoglobulin A (sIgA), lactoferrin, lysozyme, carbonic anhydrase (CA) I and II were visualised successfully in the in situ pellicle layer by gold immuno-labelling. All components were found to be distributed randomly within all layers of the pellicle. Significantly higher amounts of the proteins were detected after 120 min of formation time. Furthermore, significantly more labelled lactoferrin and lysozyme were found on buccal surfaces compared with palatal sites. For CA I, CA II and sIgA, no significant influence of the localisation was detected. All investigated anti-bacterial and buffering proteins are distributed randomly in the in situ formed pellicle layer and thus could contribute to its protective properties as an early defence barrier.

Keywords Pellicle · Lysozyme · Lactoferrin · IgA · Carbonic anhydrase

D. Deimling · C. Hannig · P. Schmitz
Department of Operative Dentistry and Periodontology,
Albert Ludwigs University of Freiburg,
Freiburg, Germany

W. Hoth-Hannig · M. Hannig (✉)
Clinic of Operative Dentistry,
Periodontology and Preventive Dentistry, Saarland University,
Building 73,
66421 Homburg/Saar, Germany
e-mail: zmkmhan@uniklinikum-saarland.de

J. Schulte-Mönting
Institute of Medical Biometry and Statistics,
Albert Ludwigs University of Freiburg,
Freiburg, Germany

Introduction

All solid surfaces exposed to the oral cavity are covered permanently by a proteinaceous layer referred to as the acquired pellicle [7, 11, 12, 17]. Studies on the composition of the acquired pellicle yield evidence for the existence of a couple of anti-bacterial proteins [1, 3, 14, 18–20, 22].

Components of both, the specific immune system and the non-specific body defences such as secretory immunoglobulin A (sIgA) [1, 3, 14, 19], lysozyme [8, 14, 18–20, 22] and lactoferrin [14, 18, 20, 22] were detected in the pellicle layer. Secretory immunoglobulin A is part of the specific immune response. Compared to the immunoglobulins in serum, sIgA does not defeat exogen particles by opsonisation. It inhibits bacterial adherence and colonisation [23]. Lysozyme and lactoferrin are part of the innate host defence system. Lysozyme destroys certain bacteria by increasing the permeability of the cell membrane [15, 26]. Besides this mechanism, lysozyme confers an additional anti-microbial property independent of its enzymatic activity. Due to its poly-cationic nature, it activates bacterial autolysins [15]. The lethal mechanism of lactoferrin is based on the growth-inhibiting influence by depriving microorganisms of its essential element iron [23].

Furthermore, buffer-active carbonic anhydrases I [22], II [18, 22] and VI [16] are present within the pellicle. These

isoenzymes participate in the maintenance of pH homeostasis [1]. Thereby this salivary enzyme family helps indirectly to protect the dental hard tissues by buffering bacterial acids [7, 27].

Even though biochemical analyses performed until today provide evidence that sIgA, lysozyme, lactoferrin and carbonic anhydrases are pellicle components, there is no information concerning their distribution within the pellicle. Therefore, the aim of the present investigation was to analyse the distribution of these anti-bacterial and buffering components within the in situ formed pellicle layer with respect to the localisation and formation time of the pellicle. Transmission electron microscopy (TEM) was chosen for visualisation of the proteins marked by gold immuno-labelling as this approach gives an insight into the integration of different proteins into the pellicle because no chemo-mechanical removal is necessary to identify the components [5].

The following hypotheses were tested:

1. Lysozyme, lactoferrin, sIgA and carboanhydrases can be visualised in the acquired in situ pellicle using an immuno-electron microscopic approach.
2. The anti-bacterial and buffering proteins are distributed randomly within the in situ formed enamel pellicle.
3. Formation time and localisation of the pellicle in the oral cavity have an impact on the amount of detectable proteins.

Materials and methods

The study design was reviewed and approved by the Ethic Committee of the Medical Association of Saarland, Germany (Proposal 52/05). All subjects gave informed written consent about their participation in the study.

Subjects

Six healthy volunteers (29–37 years old) were recruited for the in situ pellicle formation experiments. None of the subjects suffered from systemic or salivary glands diseases. None of them used any medication or had any special dietary habits. All subjects had a high level of oral hygiene and all of them were non-smokers.

Preparation of the enamel specimens and in situ pellicle formation

The pellicles were formed onto bovine enamel specimens. The round test pieces (∅=4 mm) were gained from the labial surfaces of freshly extracted bovine incisors. The incisors were extracted from cattle tested negative for BSE. The enamel slabs were flattened and wet polished by wet grinding with abrasive paper (grain sizes “1,000”, “2,400” and “4,000”). The complete removal of residues of coronal cementum was finally checked by stereomicroscopy. After the removal of the smear layer by ultrasonic cleansing with 1% NaOCl for 5 min, the specimens were disinfected in 70% ethanol for at least 30 min. The slabs were stored in bi-distilled pyrogen-free water 24 h before intra-oral exposure.

In accordance with the method published by Hannig [10, 11], the enamel specimens were exposed to the oral cavity buccally and palatally in the region of the upper first molar, using 0.3-mm thin acrylic appliances (“mini-splints”). The splints covered the crowns of the upper pre-molars and molars. The enamel slabs were mounted onto these splints by using poly-siloxane impression material (Dimension Garant L; 3M ESPE, Seefeld, Germany). Before the insertion of the appliances, the subjects just used a toothbrush and non-fluoridated floss for oral hygiene. Specimens were exposed to the oral environment for 30 and 120 min, respectively. During intra-oral exposure, volunteers refrained from eating and drinking. Each subject carried at least two specimens per localisation and exposure time, respectively.

Preparation for TEM analysis

After intra-oral exposure, the enamel slabs were rinsed with pyrogen-free water to remove non-adsorbed salivary remnants. Subsequently, the specimens were fixed in 4% paraformaldehyde/0.1% glutaraldehyde for 2 h at 4°C. Before embedding in LR White resin (London Resin, Theale, Berkshire, UK) dehydration took place in an ascending series of ethanol. The enamel part of the embedded specimens was dissolved by decalcification using 1 M HCl. Re-embedding was performed with Araldite CY 212 (Serva, Heidelberg, Germany).

A Mikrostar 45° diamond knife (Mikrotechnik, Bensheim, Germany), fixed in an Ultracut E microtome (Reichert, Heidelberg, Germany), was used to cut ultra-thin sections in series. The ultra-thin sections were mounted on 300 mesh nickel grids (Plano, Wetzlar, Germany).

Gold immuno-labelling

Visualisation of sIgA, lysozyme, lactoferrin, carbonic anhydrase I and II was performed by two-step labelling. The used antibodies are listed in Table 1. Because the primary antibodies were not conjugated with a marker, 10-nm gold-labelled secondary antibodies had to be used for visualisation by transmission electron microscopy. For detection of each of the five respective proteins, ten ultra-thin sections of each specimen were treated with one of the

Table 1 The primary and secondary antibodies used for two-step gold immuno-labelling

Protein	Primary antibody (Biotrend, Cologne, Germany)	Secondary antibody (Aurion, Wangeningen, The Netherlands)
sIgA	Anti-sIgA (human), poly-clonal, host: sheep	Anti-sheep, 10-nm gold-labelled IgG; host: rabbit
Lysozyme	Anti-lysozyme (human); poly-clonal, host: sheep	Anti-sheep, 10-nm gold-labelled IgG; host: rabbit
Lactoferrin	Anti-lactoferrin (human); poly-clonal, host: sheep	Anti-sheep, 10-nm gold-labelled IgG; host: rabbit
CA I	Anti-CAI(human); poly-clonal, host: sheep	Anti-sheep, 10-nm gold-labelled IgG; host: rabbit
CA II	Anti-CAII(human); poly-clonal, host: sheep	Anti-sheep, 10-nm gold-labelled IgG; host: rabbit

corresponding primary antibodies according to the procedure described in detail previously [5]. The ultra-thin sections were pre-treated with NH_4Cl for 5 min, washed 2 times in phosphate buffered saline (PBS) for 3 min each and stored for 10 min in 2% bovine serum albumin (BSA.) Afterwards, the specimens were incubated with the un-marked primary antibody (2 h). After rinsing with PBS (5×3 min), the sections were incubated with the 10 nm-gold labelled secondary antibodies compatible to the primary antibody (2 h). Then the samples were rinsed 5 times in PBS for 3 min each and fixed for 1 min with 1% glutaraldehyde. After fixation, the samples were washed with bi-distilled water (3×3 min) and incubated with methanolic uranyl acetate (10 min). After final washing in bi-distilled water (3×3 min), the specimens were air-dried. All steps of the protocol were performed at room temperature.

In addition, negative controls were included in each labelling experiment omitting the incubation with primary antibodies.

TEM analysis

TEM analysis was performed in a blinded manner by two investigators. The salivary pellicle was analysed in the EM 902 microscope (Zeiss, Oberkochen, Germany) at an 85,000 fold magnification. For each individual specimen, the 10-nm gold particles were counted within at least 2 randomly chosen ultra-thin sections labelled specifically with 1 of the 5 primary antibodies. The number of gold globules adhering to the pellicle was determined on a length of 100 μm. This was performed in consecutive steps of 2 μm, according to the width of the TEM screen. From the data of all 6 subjects, the mean values and standard deviations of gold particle numbers per 2-μm pellicle length were calculated for the 30-min and 120-min pellicles formed buccally and palatally, respectively. Data were analysed by two-way analysis of variance. The level of significance was set at $p<0.05$. Characteristic and representative images of the pellicle layer were photo-documented using a magnification of 85,000 fold.

Results

Structure and thickness of the in situ formed pellicles

All enamel specimens were coated by a continuous pellicle layer. Pellicle thickness depended on formation times and on the localisation of the specimens during intra-oral exposure. After 30 min, buccally exposed samples revealed a pellicle layer that ranged between 20 and 160 nm in thickness, whereas after 2 h a thickness between 20 and 350 nm was recorded. The thickness of the palatally formed pellicle varied between 5 and 30 nm after 30-min formation time and between 5 and 50 nm after 120 min, respectively.

All pellicles revealed a fine granular structure (Fig. 1).

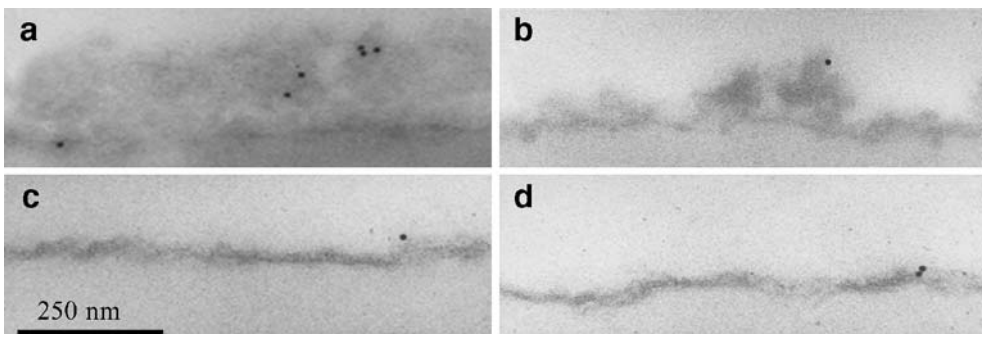

Fig. 1 Transmission electron microscopy micrographs of the in situ pellicles formed buccally (**a**, **b**) and palatally (**c**, **d**) in the region of the upper first molar for 30 min (**b**, **d**) and 120 min (**a**, **c**), respectively. All pellicles show a fine granular structure and cover the enamel surface completely with a continuous layer. The pellicle thickness depends on intra-oral exposure time and on localisation within the oral cavity. It varies between 5 nm and 350 nm. The 10-nm gold particles (*round electron-dense globules*) indicate the presence of the tested proteins: CAI in **a**; sIgA in **b**; lysozyme in **c**; lactoferrin in **d** (original magnification: 85,000, gold globule: ∅=10 nm). The enamel has been removed by hydrochloric acid treatment and thus is no longer visible at the bottom of the pellicle layer

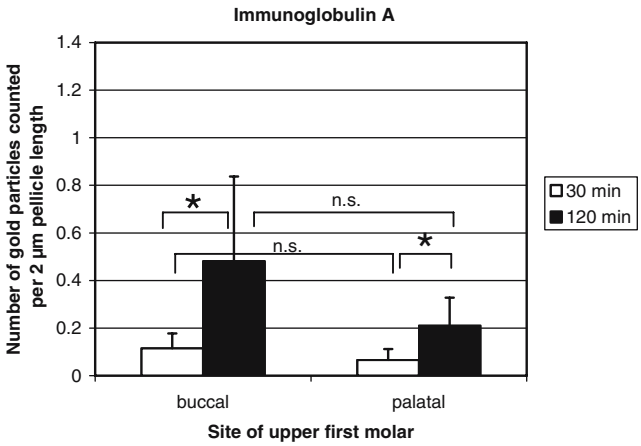

Fig. 2 Distribution of secretory immunoglobulin A within the in situ formed pellicle. *$p<0.05$, *n.s.* not significant ($p>0.05$). Enamel slabs were exposed intra-orally for 30 and 120 min on buccal and palatal sites of the upper first molar in 6 subjects

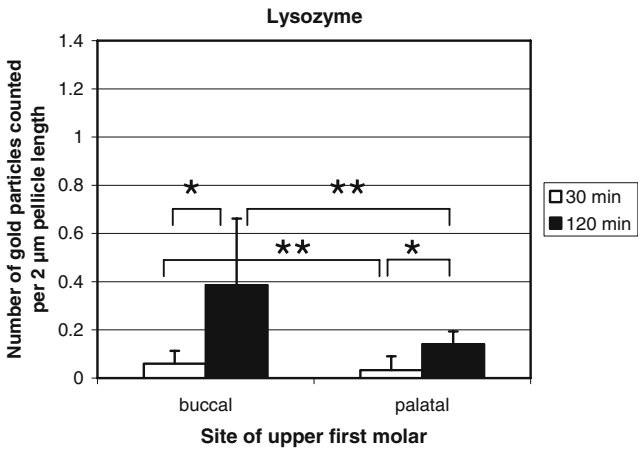

Fig. 4 Distribution of lysozyme within the in situ formed pellicle. *$p<0.05$, **$p<0.01$. Enamel slabs were exposed intra-orally for 30 and 120 min on buccal and palatal sites of the upper first molar in 6 subjects

Visualisation and distribution of sIgA, lactoferrin, lysozyme, CA I and CA II

Using the two-step gold immuno-labelling technique, the presence of sIgA, lactoferrin, lysozyme, carbonic anhydrase I and carbonic anhydrase II was visualised in all in situ formed pellicles (Figs. 2, 3, 4, 5 and 6). The analysed proteins were distributed randomly within the pellicles (Fig. 1a).

The amount of each of the gold immuno-labelled proteins increased significantly depending on the intra-oral exposure time ($p<0.05$). This was true for buccal and for palatal sites (Figs. 2, 3, 4, 5 and 6). However, the localisation of the slabs in the oral cavity had no clear impact on the number of the detected proteins. A significantly higher amount of labelled lactoferrin and lysozyme was found on buccal surfaces compared with palatal sites for 30 min and 120 min, respectively (Figs. 3 and 4). In contrast for CA I, CA II and sIgA, no significant influence of the localisation was detected (Figs. 2, 5 and 6).

Within the negative controls, only some single gold globules, adhering non-specifically to the embedding resin were found.

Discussion

In the present study, bovine enamel slabs were used for in situ pellicle formation in accordance with previous studies [2, 5, 6, 8, 10, 13]. It is easier to harvest enamel slabs of homogenous quality from bovine than from human teeth. This approach has been proven successful especially for the investigation of the activity of enzymes adsorbed in the pellicle [6, 8, 9]. In this context, it is of interest that

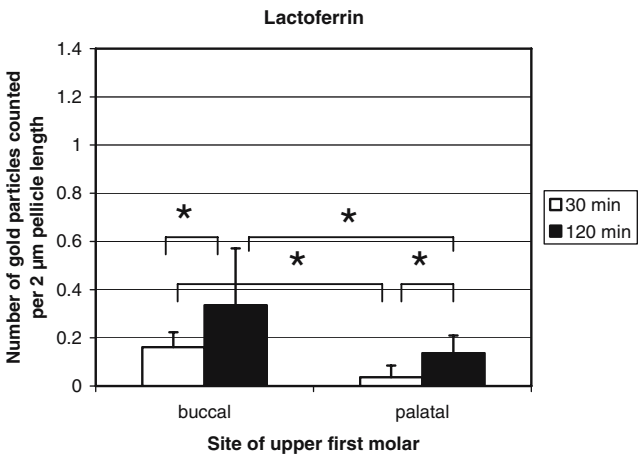

Fig. 3 Distribution of lactoferrin within the in situ formed pellicle. *$p<0.05$. Enamel slabs were exposed intra-orally for 30 and 120 min on buccal and palatal sites of the upper first molar in 6 subjects

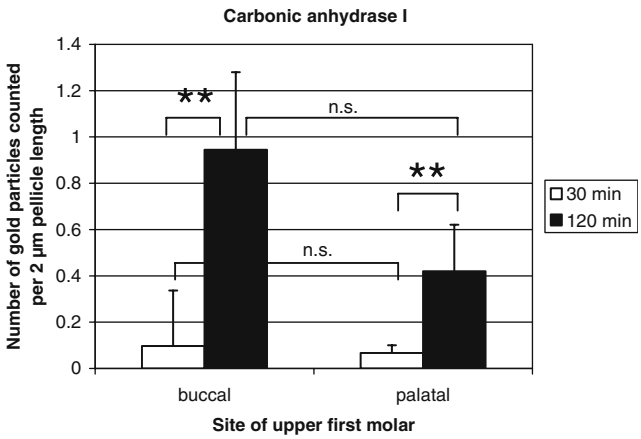

Fig. 5 Distribution of carbonic anhydrase I within the in situ formed pellicle. **$p<0.01$, *n.s.* not significant ($p>0.05$). Enamel slabs were exposed intra-orally for 30 and 120 min on buccal and palatal sites of the upper first molar in 6 subjects

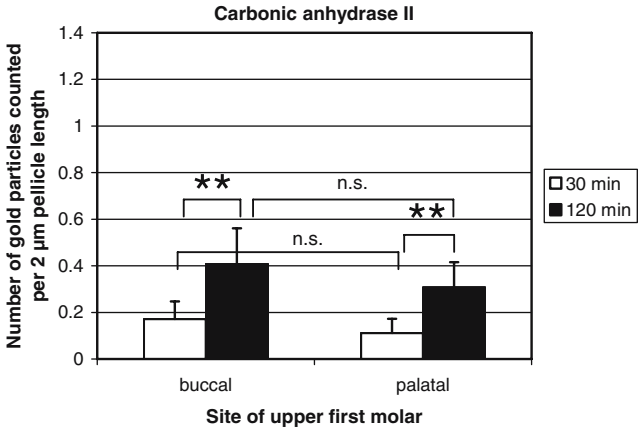

Fig. 6 Distribution of carbonic anhydrase II within the in situ formed pellicle. $^{**}p<0.01$, *n.s.* not significant. ($p>0.05$). Enamel slabs were exposed intra-orally for 30 and 120 min on buccal and palatal sites of the upper first molar in 6 subjects

different solid substrata exposed to the oral cavity have no distinct impact on immobilised enzyme activity [9].

The acrylic splints for intra-oral exposure of the enamel specimens covered only the crowns of the teeth, thus ensuring an un-hindered salivary secretion of all salivary glands, even of the palatine glands [24]. Pellicle formation took place at the buccal and at the palatal aspects of the upper first molar to get insight in the distribution of buffering and anti-bacterial components in pellicles formed in sites representative for pellicles of different thickness and ultra-structure [9, 10, 13].

The present TEM study of ultra-thin pellicle sections allowed the investigation not only of the presence of sIgA, lactoferrin, lysozyme, carbonic anhydrase I and II, but also for the first time visualised the distribution of the respective proteins within the acquired pellicle. This is possible due to the gold immuno-labelling approach in combination with TEM allowing investigation of the pellicles' components without any chemo-mechanical desorption procedure [13]. The technique is well-established for the detection of distinct proteins in the pellicle [5]. However, it should be kept in mind that not all molecules of each investigated protein are detectable by gold immuno-labelling. Some molecules or at least some of their binding sites are hidden due to the embedding procedure. Therefore, not all sites are available for the binding of the antibodies. Furthermore, the fixation procedure with paraformaldehyde and glutaraldehyde and the dehydration of the pellicle in an ascending series of ethanol before embedding might have caused conformational changes that possibly interfere with antibody binding.

Besides these methodical aspects, a reduced number of antibody-binding sites could be attributed to the conformational changes of the proteins during adsorption into the pellicle [4, 7]. For example, the main pellicle enzymes lysozyme and amylase yield increased K_m-values in the immobilised state compared to the non-immobilised state indicating a lower affinity to the substrate. This could result from conformational changes during adsorption [6, 8].

Despite these limitations, significant amounts of sIgA, lactoferrin, lysozyme, carbonic anhydrase I and II were detectable in all 30-min and 120-min in situ formed pellicle layers, respectively. This is in accordance with several other in situ studies based on different immunological or enzymatic approaches [8, 18, 22]. Besides carbonic anhydrase I and II [18, 22], carbonic anhydrase VI has been detected in in vivo formed pellicles [16]. Due to some intrinsic cross-reactivity of the CA I and CA II antibodies adopted in this study with carbonic anhydrase VI in the pellicle, some of the positively labelled proteins might be in fact carbonic anhydrase VI.

However, the present study indicates for the first time that these anti-microbial and buffering proteins are distributed randomly in all layers of the pellicle formed in situ. These findings suggest a continuous adsorption of salivary proteins during the 120 min pellicle formation time. Time-dependent increase of detected gold particles emphasises this finding. In contrast, the site of intra-oral exposure had no significant influence on the amount of CA I, CA II and sIgA marked with gold globules. It may be concluded that the localisation in the oral cavity has only limited impact on the pellicle composition despite the fact that the thickness of in situ pellicles differs considerably at different sites [5, 11] as confirmed in the present investigation. This compositional uniformity may be attributed to the adsorption of supra-molecular micelle-like structures during pellicle formation [25, 26]. Some anti-bacterial salivary proteins such as sIgA, lactoferrin and lysozyme form agglomerates with α-amylase, MG2 and proline-rich proteins. These heterotypic micelle-like complexes contribute to pellicle formation [25]. In particular, the continuous adsorption of these so-called pellicle precursors is of great relevance for establishing full biological functionality of the pellicle within very few minutes [12] as protective enzymes and molecules are exposed on the outer layer. It has been shown previously for lysozyme that this enzyme yields full enzymatic activity already after 1 min of pellicle formation [8].

Therefore, it may be speculated that also CA I, CA II, lactoferrin and sIgA remain biologically active on the surface of the pellicle and contribute to its protective properties. Further studies are required to test the biological activity of these proteins after adsorption [7]. Irrespective of their buffering and anti-microbial properties, the present investigation indicates that these biomolecules are structural components of the pellicle. Thereby, the study contributes to the understanding of the pellicle and its ultra-structure in cross-sectional views.

Concerning the tested hypotheses, it can be concluded that (1) all investigated anti-microbial and buffering

proteins are distributed randomly in the layers of the in situ pellicle irrespective of localisation and formation time and (2) the detectable amount of these proteins increases significantly with formation time.

Acknowledgement This study was supported by the German Research Foundation (Projects Ha 2718/3-1, 3-2, 3-3, Ha 5192/1-2) and by a grant from the Research Committee of the Saarland University.

References

1. Al-Hashimi I, Levine MJ (1989) Characterization of in vivo salivary-derived enamel pellicle. Arch Oral Biol 34:289–295
2. Amaechi BT, Higham SM, Edgar WM, Milosevic A (1999) Thickness of acquired salivary pellicle as a determinant of the sites of dental erosion. J Dent Res 78:1821–1828
3. Carlen A, Borjesson AC, Nikdel K, Olsson J (1998) Composition of pellicles formed in vivo on tooth surfaces in different parts of the dentition, and in vitro on hydroxyapatite. Caries Res 32:447–455
4. Cserhati T, Szogyi M (1995) Role of hydrophobic and hydrophilic forces in peptide–protein interaction: new advances. Peptides 16:165–173
5. Deimling D, Breschi L, Hoth-Hannig W, Ruggeri A, Hannig C, Nekrashevych Y, Prati C, Hannig M (2004) Electron microscopic detection of salivary alpha-amylase in the pellicle formed in situ. Eur J Oral Sci 112:503–509
6. Hannig C, Attin T, Hannig M, Henze E, Brinkmann K, Zech R (2004) Immobilisation and activity of human alpha-amylase in the acquired enamel pellicle. Arch Oral Biol 49:469–475
7. Hannig C, Hannig M, Attin T (2005) Enzymes in the acquired enamel pellicle. Eur J Oral Sci 113:2–13
8. Hannig C, Hoch J, Becker K, Hannig M, Attin T (2005) Lysozyme activity in the initially formed in situ pellicle. Arch Oral Biol 50:821–828
9. Hannig C, Wasser M, Becker K, Hannig M, Huber K, Attin T (2006) Influence of different restorative materials on lysozyme and amylase activity of the salivary pellicle in situ. J Biomed Mater Res A 78:755–761
10. Hannig M (1997) Transmission electron microscopic study of in vivo pellicle formation on dental restorative materials. Eur J Oral Sci 105:422–433
11. Hannig M (1999) Ultrastructural investigation of pellicle morphogenesis at two different intra-oral sites during a 24-h period. Clin Oral Investig 3:88–95
12. Hannig M, Joiner A (2006) The structure, function and properties of the acquired pellicle. In: Duckworth RM (ed) The teeth and their environment. Monogr Oral Sci, Basel, Karger pp 29–64
13. Hannig M, Khanafer AK, Hoth-Hannig F, Al-Marrawi F, Acil Y (2005) Transmission electron microscopy comparison of methods for collecting in situ formed enamel pellicle. Clin Oral Investig 9:30–37
14. Kraus FW, Orstavik D, Hurst DC, Cook CH (1973) The acquired pellicle: variability and subject-dependence of specific proteins. J Oral Pathol 2:165–173
15. Laible NJ, Germaine GR (1985) Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect Immun 48:720–728
16. Leinonen J, Kivela J, Parkkila S, Parkkila AK, Rajaniemi H (1999) Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res 33:185–190
17. Lendenmann U, Grogan J, Oppenheim FG (2000) Saliva and dental pellicle—a review. Adv Dent Res 14:22–28
18. Li J, Helmerhorst EJ, Troxler RF, Oppenheim FG (2004) Identification of in vivo pellicle constituents by analysis of serum immune responses. J Dent Res 83:60–64
19. Orstavik D, Kraus FW. The acquired pellicle (1973) immunofluorescent demonstration of specific proteins. J Oral Pathol 2:68–76
20. Rüdiger SG, Carlen A, Meurman JH, Kari K, Olsson J (2002) Dental biofilms at healthy and inflamed gingival margins. J Clin Periodontol 29:524–530
21. Soares RV, Lin T, Siqueira CC, Bruno LS, Li X, Oppenheim FG, Offner G, Troxler RF (2004) Salivary micelles: identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycosylated proline-rich protein and lysozyme. Arch Oral Biol 49:337–343
22. Vacca Smith AM, Bowen WH (2000) In situ studies of pellicle formation on hydroxyapatite discs. Arch Oral Biol 45:277–291
23. Van Nieuw Amerongen A, Bolscher JG, Veerman EC (2004) Salivary proteins: protective and diagnostic value in cariology? Caries Res 38:247–253
24. Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV (1996) Human glandular salivas: their separate collection and analysis. Eur J Oral Sci 104:346–352
25. Vitkov L, Hannig M, Nekrashevych Y, Krautgartner WD (2004) Supramolecular pellicle precursors. Eur J Oral Sci 112:320–325
26. Wang YB, Germaine GR (1993) Effects of pH, potassium, magnesium, and bacterial growth phase on lysozyme inhibition of glucose fermentation by *Streptococcus mutans* 10449. J Dent Res 72:907–911
27. Wolinsky L (1994) Caries and cariology. In: Nisengard R, Newman M (eds) Oral microbiology and immunology. Saunders, Philadelphia, pp 341–359