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REVIEW

# The oral cavity—a key system to understand substratum-dependent bioadhesion on solid surfaces in man

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**Abstract** One of the greatest challenges in life sciences and biomaterials research is adhesion of biomolecules and bacteria to solid surfaces in aqueous solutions. An example concerning everybody is biofilm formation in the oral cavity on dental materials and dental hard substances, respectively. The main characteristics typical for any bioadhesion can be observed excellently in the oral cavity. Initially, a proteinaceous layer termed pellicle is formed. It mediates the interactions between solid substrata, oral fluids and microorganisms. Numerous different materials with differing physico-chemical properties and possible impact on the acquired pellicle are present in the oral cavity such as enamel, dentine, restorative materials or dental implants. Despite the fact that in vitro studies demonstrate considerable differences of experimental pellicles formed on these materials, the in situ pellicles seem to be relatively similar and level off the different properties of the underlying substrates. However, the bacterial colonisation of pellicle-coated surfaces under in vivo conditions differs considerably. Long-range forces and detachment of biofilm layers may account for this phenomenon despite the masking effect of the pellicle. Accordingly, low-energy surfaces are desirable for restorative materials exposed to the oral cavity to minimise bacterial adhesion. The oral cavity is an easy accessible in vivo model for understanding bioadhesion and for investigation of protein–surface interactions noninvasively. For evaluation of biofilm formation on dental materials, in situ or in vivo studies are preferable.

**Keywords** Dental materials · Pellicle · Biofilm · Oral cavity · Protein adsorption

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## The ubiquitous phenomenon of bioadhesion

Natural scientists, medical scientists and engineers of all branches are faced with the ubiquitous phenomenon of bioadhesion and biofilm formation on different solid surfaces. This is relevant for hulks of supertankers as well as for catheters, water pipes or contact lenses [58, 78, 83, 84].

These interfacial phenomena have become an integral part of all human endeavours, since no conceivable systems can exist without the coexistence of different states of matter [93–95]. The solid–liquid interface is of paramount importance due to its involvement in many biological processes [82–84, 93–95]. Also, in modern dentistry, a vast number of different materials are adopted and therewith an object of bioadhesion. In the oral cavity, caries, peri-implantitis and periodontitis are caused by oral biofilms formed on the initial proteinaceous coating of all solid substrata, the acquired pellicle [53, 54, 70, 82–85]. In this context, it is noteworthy that in contrast to all other human tissues, dental hard substances are non-shedding surfaces [143]. Accordingly, intraoral biofilm management is strongly required. However, anti-adhesive easy-to-clean or self-cleaning surfaces are not only demanded for dental application but also in other medical disciplines, for example in ophthalmology for intracorneal implants or

intraocular lenses, respectively [78]. Ophthalmology is also faced with the problem of bacterial adherence in a wet chamber open to external influences. The development of extended wear contact lenses requires materials that will selectively bind specific proteins to minimise the bacterial adhesion [78]. This example demonstrates clearly that interdisciplinary research projects of medics, material scientists, biochemists and dentists will yield new intelligent materials. The oral cavity is an often overlooked easy accessible and relevant in vivo model for noninvasive monitoring of substrate-dependent bioadhesion. The objective of this review was to summarise our understanding of the role of the salivary protein conditioning film in the process of bacterial adhesion and biofilm formation on biomaterials under oral conditions. Thereby, it was intended to elucidate to which extent bioadhesion is substrate-dependent in the oral cavity.

## Physico-chemical determinants of bioadhesion

At solid–liquid interfaces, the fundamental of a spontaneous adsorption process is that more energy is released than gained according to the Gibbs law of free energy, meaning a gain in entropy or a decrease in the enthalpy, respectively [93–95]. This is described by the equation [93–95]:

$$\Delta_{ads}G = \Delta_{ads}H - T\ \Delta_{ads}S < 0$$

where $G$ = Gibbs free energy, $H$ = enthalpy, $T$ = absolute temperature, $S$ = entropy and $\Delta_{ads}$ = net change of the thermodynamical parameters.

Adsorption of proteins from an aqueous solution onto a solid surface is the result of various types of interactions that simultaneously occur between all the components, namely the fluid, the solid and the solubilised proteins. The polarity of each of these components of course has great impact on the adsorption process. The polarity is reflected in the hydrophilicity or hydrophobicity of the interacting components.

The effective forces relevant for protein adsorption can be divided according to the range of the interactions. Van der Waals forces and Coulomb forces represent the main exponents of the long-range forces (50–100 nm). Hydrophobic interactions, the most relevant forces in water, are of medium range (10–50 nm) [152]. Electrostatic interactions, ionic interactions and Lewis acid–base interactions are classified as short-range interactions as well as covalent bonds or hydrogenic bonds (less than 5 nm). These forces of different range have an impact on protein adsorption and on protein conformation after adsorption (Fig. 1) [94–96, 106, 143]. However, the acid–base interactions or electron acceptor/electron donor interactions are regarded as the most predominant non-covalent forces [152].

Superordinate physical variables are helpful to describe the surface properties. The surface free energy (mJ/m$^2$) is a physical value describing the whole energy of a solid surface as an equivalent to the surface tension of a fluid. The contact angle reflects the interactions of fluids with solid surfaces depending on the polarity, hydrophilicity and wettability of the involved components [21, 33]. The contact angle is a function of the surface energies of a system [59]. The surface free energy, $\gamma_s$, its polar, $\gamma_s^p$, and dispersion components, $\gamma_s^d$, are calculated from contact angle measurements [22, 33]. High water contact angles mean poor wettability [33, 115].

The current models for initial bioadhesion were described thoroughly by Teughels et al. [143] in a recent review. Initial adhesion of bacteria is characterised by weak and reversible adhesion mediated through long- and short-range forces (Figs. 1 and 2).

The thermodynamic model regards the surface free energy as the main determinant and does not differentiate electrostatic interactions. The alteration of Gibbs free energy due to adsorption of the bacteria is described as $\Delta G = \gamma_{sb} - \gamma_{s1} - \gamma_{b1}$. Thereby, $\gamma_{sb}$ represents the surface–bacterium interfacial free energy, $\gamma_{sl}$ the surface–liquid interfacial free energy and, $\gamma_{bl}$ the bacterium–liquid interfacial free energy [1, 8, 143]. Adhesion takes place if the equation is negative, as a minimisation of energy is favoured in the nature.

The DLVO model named after Derjaguin, Landau, Verwey and Overbeck depicts the interaction of a negatively charged bacterium with a solid surface in aqueous solutions (Fig. 3). Both the bacterium and the surface are coated by an electrical double layer named Stern layer; the outer surface of this layer is charged negatively. Two forces are relevant for the interaction—the Lifshitz–van der Waals attractive forces, becoming active at a range of even more than 50 nm or less, and the electrostatic repulsive forces of the similarly charged surfaces. The Gibbs energy of the interaction is calculated as a function of the distance, summing up repulsive and attractive forces when the electric double layers of the respective forces overlap (Fig. 1) [13, 110, 143, 151]. The hydrophobic interactions, quantitatively the strongest effect in water, are always attractive [152]. In contrast, the acid–base interactions neglected in the classical DLVO theory can be either attractive or repulsive depending on the degree of hydrophilicity or hydrophobicity of the surfaces involved [152]. Accordingly, an extended DLVO theory (XDLVO) was described by van Oss et al. combining the classical DLVO model with thermodynamic aspects [143, 151, 152]. In water, the acid–base interactions are to be considered as they represent about 90% of the total non-covalent interaction forces, either attractive or repulsive [152]. The XDLVO approach intends to combine four elementary non-

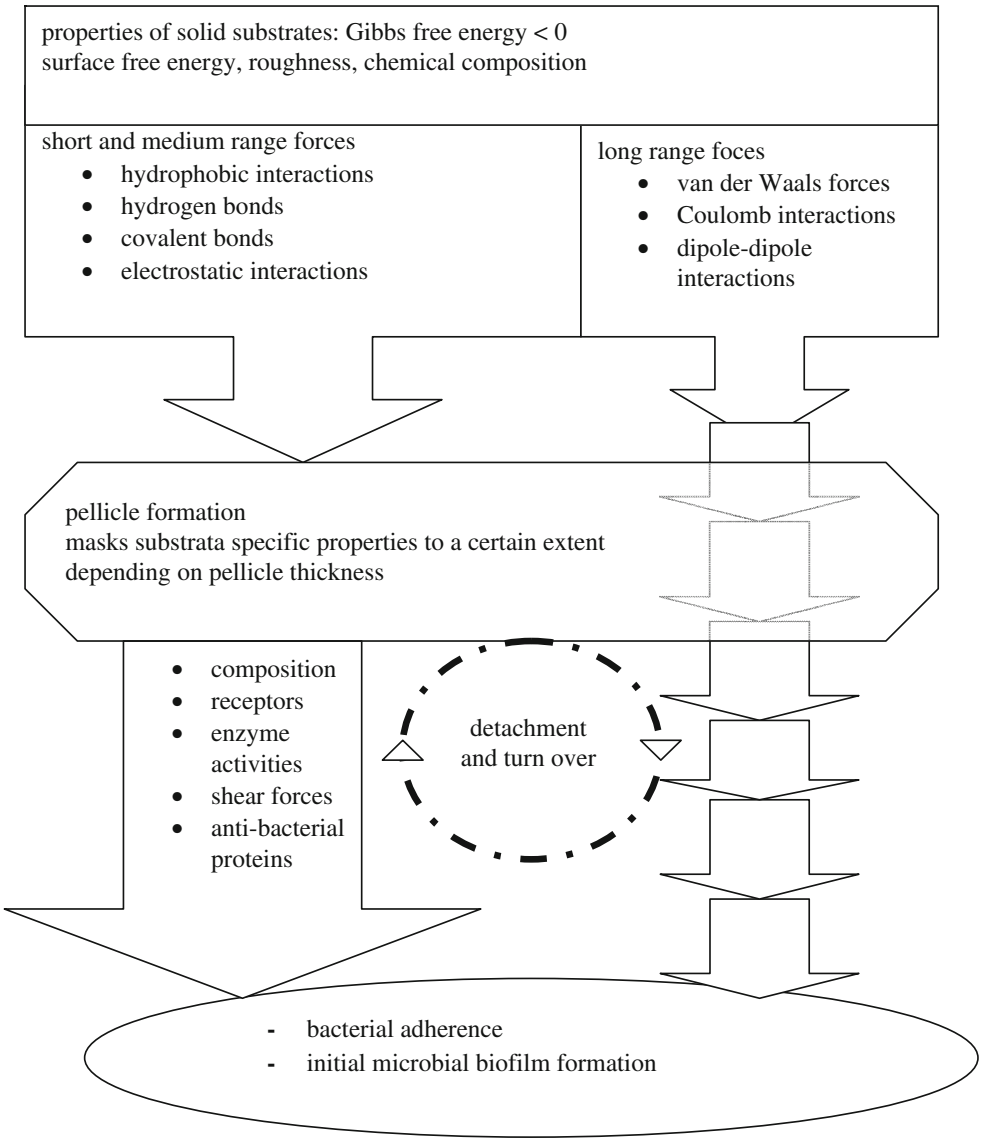


**Fig. 1** Interactions of proteins, fluids and bacteria in the oral cavity. Substrate-specific surface properties are masked by the pellicle to a certain extent, but biofilm formation is influenced by long-range forces transferred through the pellicle layer

covalent forces: van der Waals forces, electrostatic forces, Brownian molecular motion and acid–base interactions [77, 143, 151]. Each of the three non-covalent interactions decays depending on the respective thermodynamic circumstances as a function of distance [152]. The acid–base interactions based on electron donating and accepting molecules are more pronounced than the other forces, but are relatively short ranged [143, 152]. However, hydrophilic repulsion may occur if hydrophilic particles attract water molecules more strongly than the acid–base cohesive attraction between water molecules [152].

In the oral cavity, there are no pure chemical substances but a proteinaceous cocktail with colloidal components of various properties. Accordingly, a colloid chemical approach is helpful for interpretation of the phenomena occurring during pellicle formation in the oral cavity [93–95]. The electrostatic properties of colloidal particles or protein agglomerates in a suspension are described by the zeta potential. The zeta potential refers to the electrostatic potential generated by the accumulation of ions at the surface of the colloidal particle. The ions are arranged in an electrical double layer consisting of the previously described Stern layer and the diffuse layer [93–95, 160].

Protein adsorption is characterised by a reorientation and structural rearrangement after first adhesion to the surface resulting in conformational changes [38]. The charged groups redistribute according to the polarity of the surface. Changes in the hydration of the sorbent and the protein surface are the result [93–95].

Low structural stability of protein aggregates facilitates adsorption of proteins to a surface due to easier structural rearrangements. The rearrangement of the proteins may continue for a long period of time [95]. The degree of structural rearrangements depends on the rate of deposition

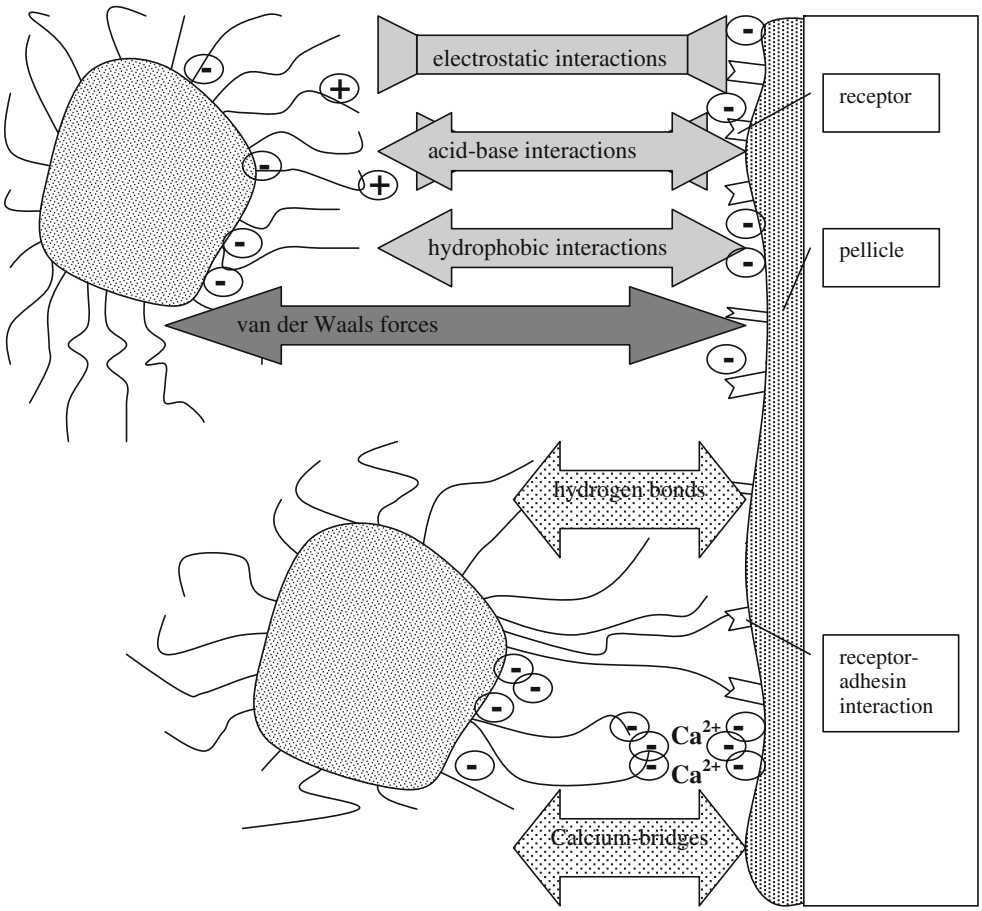


**Fig. 2** Initial bacterial approach and adhesion to a pellicle-coated surface is mediated by fimbriae as well as by long-, medium- and short-range forces and leads to specific receptor–adhesin interactions. The forces may be attractive (hydrophobic interactions, van der Waals forces, hydrogen bonds, calcium bridges), repulsive (electrostatic interactions) or both (acid–base interactions)

relative to the rate of structural changes [150, 159]. Such long-term structural alterations involve an expanding contact between the single protein molecule and the sorbent surface. This may cause displacement of the later adsorbed neighbouring molecules [93–95]. It was described for plasma that upon its contact with surfaces, at least five proteins in succession displace each other within 1 min [158]. Such effects might also be of relevance for pellicle formation.

The process of protein adsorption is multifactorial, as isoelectric point, repulsion, desorption, structural stability, heterogeneity of the sorbent and the adsorbed protein layer as well as several orientations and conformations of the adsorbed protein layer have an impact [22, 96]. In the oral cavity, these mechanisms are superimposed by bacterial colonisation. The redistribution of the charged groups is modulated by low-molecular-weight ions, by the pH of the surrounding solution and by the isoelectric point. The isoelectric point (p*I*) is the pH at which a protein carries no net electric charge.

For the surface of hydrophilic sorbents, hydration is favourable and the hydration opposes the adsorption. If adsorption occurs, some hydration water will be retained. On the other hand, if the surface is hydrophobic, dehydration of that solid would stimulate protein adsorption, as this means a gain in entropy [93–95].

Also, the hydrophobicity of the protein exterior influences the protein adsorption.

Under quiescent conditions, proteins reach the surfaces by Brownian molecular motion in a stochastic manner. However, in the oral cavity, saliva is oversaturated with the proteins and the proteins adsorb from a flowing solution. Due to the highly varying conditions with the inhomogenous adsorption solution saliva, it is not sensible to use mathematical equations as they can only offer an approximative approach but do not completely describe the complex process of bioadhesion. The adsorption is smaller than the flux to the surface, in general due to the electrostatic repulsion or thermodynamic effects or because a fraction of proteins does not collide in the proper orientation that is required for attachment to the surface [93–95].

## The process of bioadhesion and biofilm formation in the oral cavity

The acquired pellicle was defined as initial integument on oral surfaces which is free of bacteria [20, 54]. This proteinaceous layer is composed of adsorbed proteins, amongst them several enzymes, glycoproteins and other macromolecules [40, 53, 67, 70, 72]. Despite the versatile

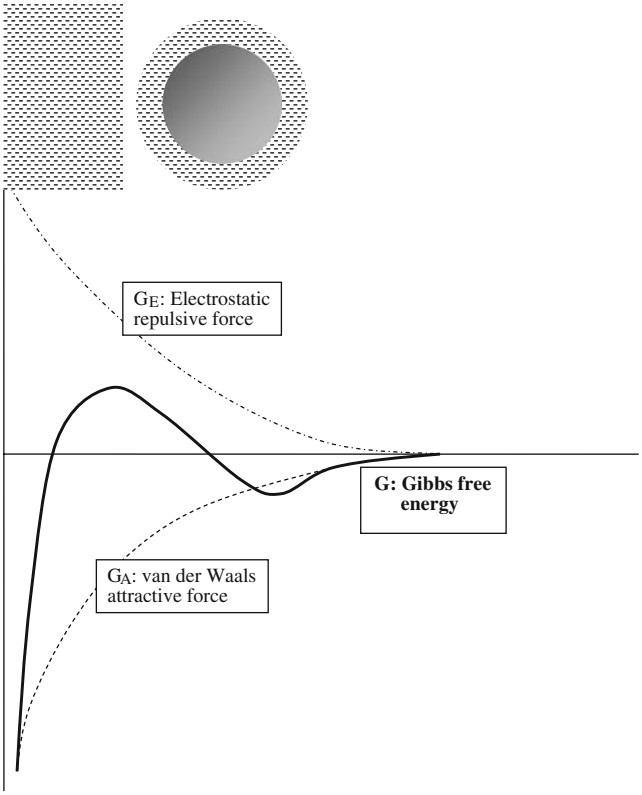


**Fig. 3** DLVO theory. Interaction between negatively coated surface and negatively coated bacterium or protein aggregates, respectively. There are attractive forces (Lifshitz–van der Waals forces) active at a distance of 50 nm and electrostatic repulsive forces. These repulsive forces are caused by the Stern layer formed in aqueous solutions. The resulting Gibbs free energy is a resultant of both. The hydrophobic interactions are always attractive [152]. Figure modified after Teughels et al. [143]

oral fluids, pellicle formation is of high selectivity, since only a fraction of the proteins present in the saliva is found in the pellicle [47, 53, 56, 168]. The first pellicle formation occurs almost instantaneously [22, 30, 49, 147]. So-called pellicle precursor proteins, phosphoproteins with high affinity to hydroxyapatite are the first to adsorb to the tooth surface. Examples are statherin, histatin and proline-rich proteins [53, 57]. This interaction is conveyed by the ionic calcium and phosphate layer at the enamel surface [9, 53, 57, 88]. Due to this fact, surface-specific protein adsorption to restorative surfaces is conceivable. However, besides ionic interactions, van der Waals forces and hydrophobic interactions contribute to the formation of the proteinaceous pellicle layer [53, 153, 156]. The adsorption of proteins, thereby replacing structured water molecules at the surface, means a gain in entropy as a driving force for pellicle formation [53, 153, 156]. The first adsorbing proteins undergo an unfolding and flattening process, thereby increasing their cross-sectional area and providing rapid surface coverage [95]. This process is visualised as the electron dense basal layer observed in transmission electron microscopy (TEM) micrographs (Figs. 4, 5) [24, 48, 49]. The rapid first stage is followed by a second phase characterised by a continuous adsorption of biomolecules from the oral fluids [53]. Thereby, the pellicle formation reaches a plateau [68, 132, 137]. Protein aggregates rather than single molecules are responsible for the fast increase of the pellicle's thickness within 30–90 min. These micelle-like globules and heterotypic complexes are described as supramolecular pellicle precursors [112, 120, 157]. Most of the parotid proteins are secreted in globular aggregates with a diameter of approximately 150 nm [112, 120, 157]. The amino acid profile of these structures is similar to that of 2-h in vivo pellicles [170]. Furthermore, these aggregates have a negatively charged surface layer coating a hydrophobic interior [112, 120]. Accordingly, their adsorption process may be explained with the DLVO theory, and a masking of material-specific surface properties is conceivable [143].

The function of the pellicle is ambivalent. On the one hand, the pellicle serves as a lubricant and as an anti-erosive barrier und buffer [43, 52, 53]. In addition, the antibacterial proteins lactoferrin, cystatins and lysozyme add protective properties [25, 41, 46, 53, 105, 147]. Even sIgA was detected in the pellicle [4, 15, 17, 25, 72]. On the other hand, the pellicle features some properties facilitating bacterial adhesion. Several pellicle components such as amylase, proline-rich proteins, Mucin MG 2, fibrinogen and lysozyme serve as specific receptors for bacterial adherence [26, 53, 54, 111, 121, 122]. Initial bacterial adhesion passes through a phase of weak and reversible binding before an irreversible attachment is established (Fig. 2) [73, 83, 84]. Reversible initial binding occurs preferentially in the surface irregularities where microorganisms are protected against mechanical shear forces (Fig. 4) [10, 16, 97, 110, 142].

It is reasonable to suppose that composition, formation, ultrastructure, functionality and bacterial colonisation of the initial oral biofilm may be modulated by the underlying substrata. Dental research is faced with a vast number of materials with different physical and chemical properties exposed to the oral fluids: dental hard substances, ceramics, titanium, resin composites, nanomaterials, gold alloys, denture base materials, orthodontic brackets or suture materials [29, 69, 92, 101]. It is desirable to achieve minimal colonisation of these materials with pathogenic bacteria. Thus, the aim of the present review was to depict the possible impact of different solid substrata on the pellicle and on the initial oral biofilm. Thereby, it was to be evaluated if the pellicle masks the physico-chemical characteristics of solid substrata exposed to the oral fluids or if the surface properties are transferred to impact the bacterial biofilm.

There are many rather inconsistent and incomparable studies on initial biofilm formation on different solid substrata performed with very different methodical approaches. Three different types of studies are to be

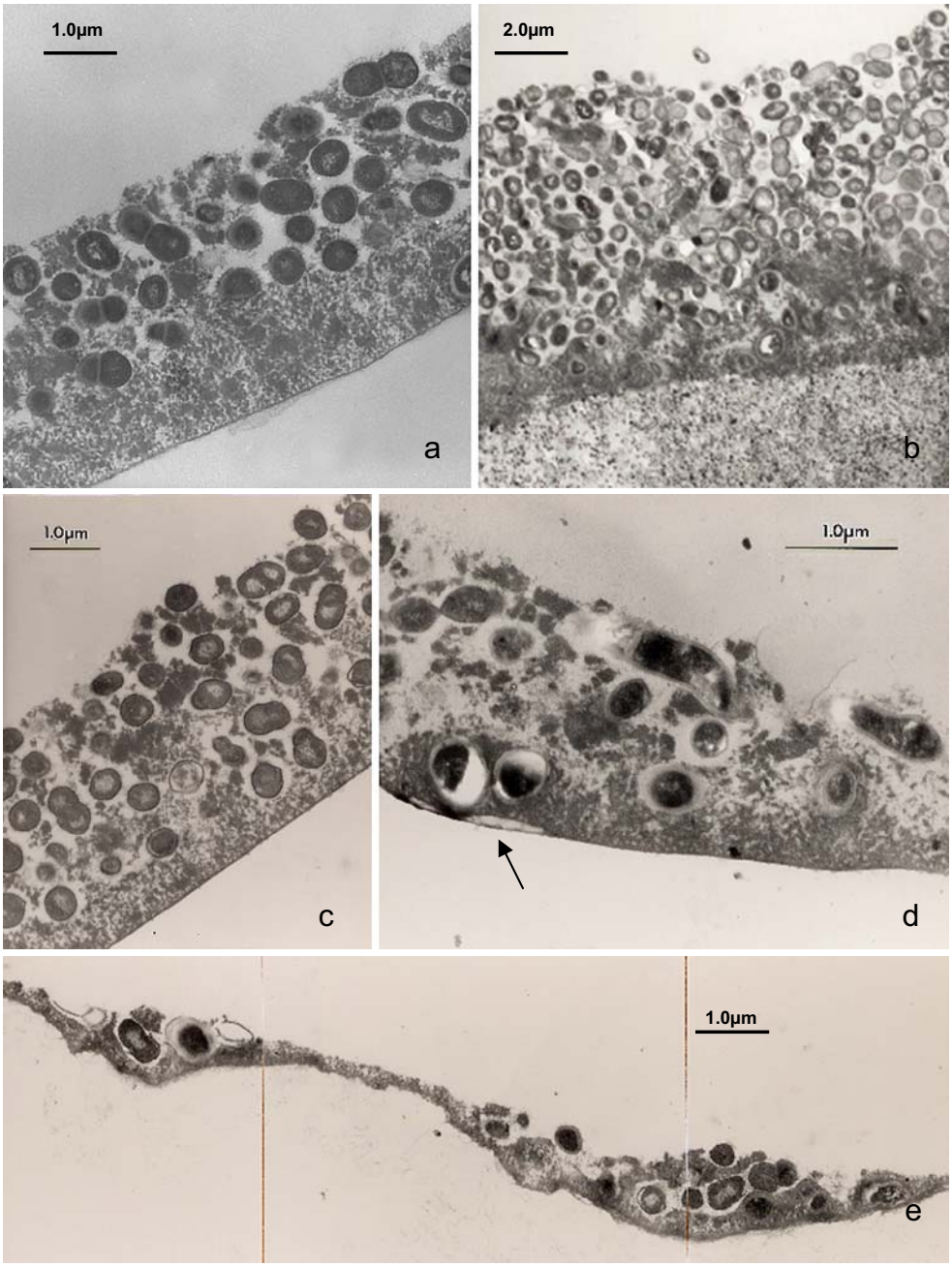


**Fig. 4** Biofilms formed in situ over periods of 24 h on buccally positioned specimens of enamel (**a**), resin composite (**b**), polished ceramics (**c**), glazed ceramics (**d**) and on a lingually positioned enamel specimen (**e**). Due to shear forces acting on the surface of the lingually placed specimen, the respective biofilm is significantly thinner and microorganisms are only detectable in surface irregularities. However, the biofilms on the different substrata are of high ultrastructural uniformity. Original magnifications of the TEM micrographs: **a** 12,000-fold; **b** 3,000-fold; **c** 10,000-fold; **d** 15,000-fold; **e** 7,000-fold. During the preparation process for TEM analysis, enamel and ceramics were dissolved by acid treatment and thus are not visible anymore. Note partial detachment of the biofilm from the ceramic sample at the interface between the basal pellicle layer and the adhering proteinaceous layer (**d**, *arrow*)

differentiated: in vivo studies (the biofilm is scrapped of the tooth or restoration, respectively, in the oral cavity), in situ studies (specimens of the certain substrata are exposed in the oral cavity for a period of time) and in vitro studies (different substrata are exposed to collected saliva or adsorption solutions extraorally) [86]. Notwithstanding, bioadhesion is a complex process dependent on a vast number of factors [93–95, 143]. Hence, it seems tempting to simulate and to monitor distinct aspects in laboratory setups or in vitro, respectively, to eliminate sources of irritation [87]. However, it is questionable if these in vitro studies mirror the intraoral in vivo situation. Accordingly, it is to be verified if in vitro studies are appropriate for understanding bioadhesion in life sciences or if they misdirect.

## Substratum-dependent protein adsorption and biofilm formation on dental materials in vitro—appropriate for understanding bioadhesion in life sciences?

### Protein adsorption to different surfaces in vitro

The first adhesion of proteins is governed by physicochemical interactions rather than specific bonds [34, 155].

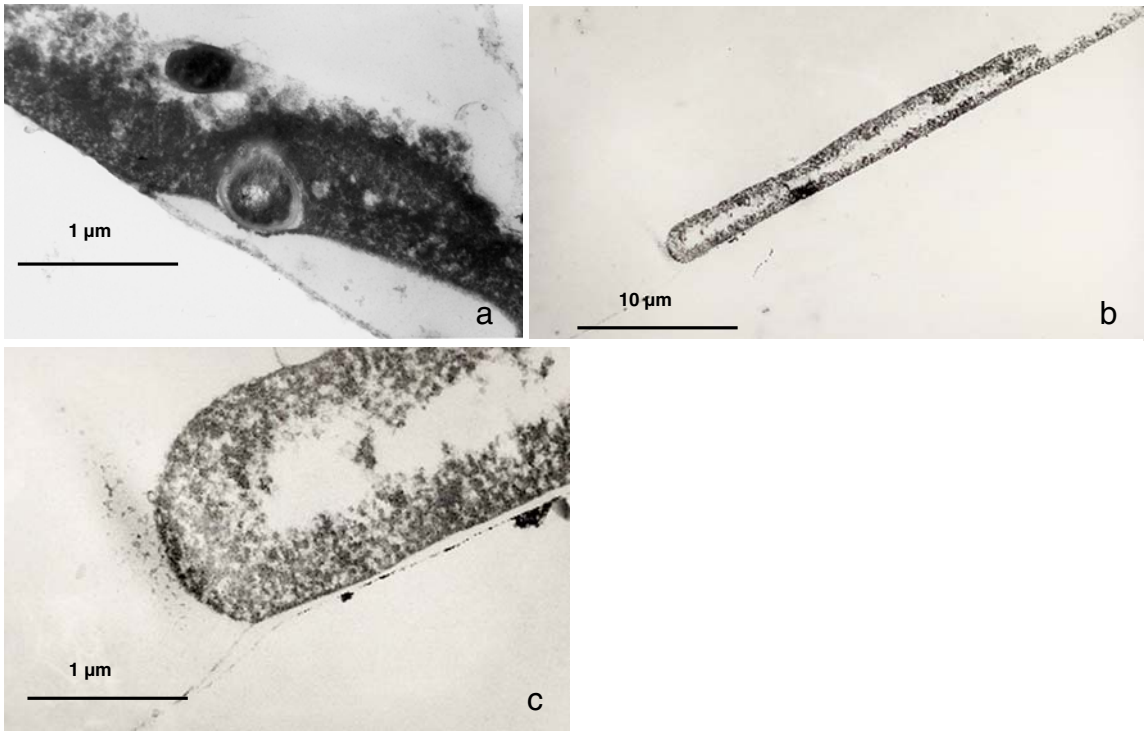


**Fig. 5** Detachment phenomena observed in situ within the adherent biofilm or mature pellicle layer: 6-h in situ pellicle and biofilm formation on a ceramic specimen (**a**) and 24-h in situ biofilm formation on a nanocomposite coated titanium specimen (**b**, **c**). The coated titanium as well as the ceramic have been completely dissolved due to treatment with hydrofluoric acid. On top of the dissolved surface, a thin condensed layer of adsorbed salivary biopolymers can be detected. The globular layer of adsorbed salivary proteins with adherent bacteria is visualised in the state of detachment from the basal layer. The observed phenomenon offers an explanation for the minor bacterial colonisation of glazed ceramic materials or low-energy surface coatings, respectively. Original magnifications of the TEM micrographs: **a**, **c** 30,000-fold; **b** 7,000-fold

Most in vitro studies indicate a great impact of these different physical surface characteristics on protein adsorption [5, 18, 69, 74, 75, 154]. Thereby, it is noteworthy that protein layers on samples rinsed with water are thicker as compared with unrinsed samples [34]. This indicates that the hydration layer has considerable impact on protein adsorption.

Proteins have a different affinity to diverse substrata. More proteins adsorbed to rough surfaces [16, 90]. In an in vitro scanning force microscopic approach, the adhesion forces and the adsorption process of bovine serum albumin onto enamel and a compomer were monitored in real time. As expected, different modes of adhesion were observed [124]. In vitro exposed Germanium prisms with high or low surface free energy featured a pellicle formation of certain qualitative selectivity as studied by atomic force microscopy. The amount of adsorbed protein was correlated with the surface energy [5]. In another study, the adhered protein mass in films on medium energy surfaces was higher than on low-energy surfaces [18]. In addition, it has been shown that modification of surfaces such as silicon wafers by coating with polyethylene glycol or fluorocarbon moieties reduces the protein adsorption [90].

Several studies investigated protein sorption to hydrophilic and hydrophobic surfaces. Higher amounts of salivary proteins adsorbed on hydrophobic surfaces as compared with hydrophilic samples [74–76, 154–156]. Under in vitro conditions, proteins mostly have a higher affinity to polymer materials, which have usually a hydrophobic character [140]. Hydrophobic interactions seem to play an important role as a driving force in pellicle formation [74–76, 154–156]. Hydrophilic surfaces interact preferentially with polar groups of adhering substrates, whereas adsorption to hydrophobic surfaces is assumed to be mediated by the hydrocarbon tail of organic molecules [155]. Larger amounts of hydrophobic salivary fractions adsorb on hydrophobic surfaces than on hydrophilic materials. On hydrophilic surfaces, the largest amounts from a more hydrophilic high-molecular-weight fraction are adsorbed [155]. It was concluded that the substrate-dependent adsorption behaviour of salivary proteins might show a wide variation among the different fractions [75, 88, 155]. This is supported by several studies on the adsorption of specific proteins. Different adsorption patterns were observed for statherin and proline-rich proteins of different hydrophobicity to hydrophobic and hydrophilic surfaces

[76]. In a study with glass, polytetrafluoroethylene and methacrylate, highest adsorption of albumin was observed on the most hydrophobic surface [146]. More proteins such as lysozyme, proline-rich proteins or cysteine-containing proteins are adsorbed to the enamel than to cementum [114]. In contrast, another study found twice as many potential protein binding sites for cementum versus enamel [31]. However, salivary proteins adsorbed on the hydrophobic specimens were more loosely associated and a lager fraction could be desorbed [156].

Besides critical surface tension, dispersions and polar contributions to the surface energy, other chemical surface characteristics were assumed to be responsible for the pronounced differences of protein adsorption such as corrosion of the materials or calcium pretreatment [87, 139]. This is confirmed by the influence of calcium on albumin sorption or of fluoride application on lysozyme and albumin sorption to enamel [31, 61]. Despite the high number of in vitro studies yielding an influence of different solid substrata on adsorption of biomolecules, some investigations found quite identical patterns of protein adsorption to differently pretreated hydroxyapatite surfaces or to surfaces of different hydrophobicity, respectively [30, 163]. Not only in experimental pellicles on resin [169] but also on other materials such as titanium or orthodontic brackets were the typical components of the acquired enamel pellicle such as amylase, proline-rich proteins or mucin detected [3, 28].

## Bacterial colonisation of different materials in vitro

In general, most in vitro setups indicate a strong impact of different physical surface properties on the bacterial adhesion and colonisation. With and without a pellicle, bacterial adhesion and biofilm formation as well as biofilm resistance in vitro correlate with surface roughness [16, 91, 110, 142, 145] and polishing reduces the number of adhering bacteria [62]. Irrespective of the surface roughness, least amount of plaque adhere to ceramics, generally regarded as low-adhesive materials [62, 87]. Bacterial adhesion to a bare material of known surface free energy can be well estimated on the basis of the interfacial thermodynamics [104]. However, adhesion to protein-coated surfaces is much more complex. Pellicle coating itself results in reduced numbers of adhering bacteria and has a homogenizing effect on the surface free energy in vitro [3, 28, 91, 104, 125, 127, 141, 160]. In addition, experimental salivary pellicles reduce the differences of bacterial colonisation among uncoated materials of varying physico-chemical properties [91, 103, 104]. However, protein adsorption does not fully abolish the influence of the chemical surface characteristics [91]. It was shown that despite the reduction of bacterial adherence due to pellicle formation, the tendency of all bacterial strains toward negative slopes persisted [104]. This was to be expected on the basis of surface thermodynamics of the bare substrata and may indicate that at least to a certain extent, substratum properties are transferred from the substratum–protein interface to the protein–bacteria interface [104]. This conclusion was drawn earlier for streptococcal adhesion to bovine-serum-albumin-coated artificial solid substrata in vitro [103]. The transfer of the surface properties is less pronounced by a maturated 2-h in vitro pellicle than by a 5-min pellicle [104].

In another in vitro approach, different amounts of bacteria adhered on different gold alloys [99]. This observation gave the reason to believe that although the known difference in chemical composition among pellicles formed on different materials does not imply striking differences in protein adsorption, they may still be of importance for the selectivity of bacterial adhesion and growth. In the presence of an experimental salivary pellicle, ceramic and fibre-reinforced composites bound more bacteria than the other materials confirming the hypothesis [142]. Interestingly, an up to 20-fold difference in the binding of some bacterial strains to different surfaces—such as titanium, polymeric substrata or enamel—was recorded [160, 164].

Modulation of early *Streptococcus sobrinus* biofilm formation on various dental restorative materials by typical salivary proteins was also investigated [140]. Highest bacterial colonisation was observed onto polymer materials. This was mediated by amylase and albumin, typical bacterial receptors exposed in high numbers on the respective substrata [140]. Thereby, it is noteworthy that different bacterial strains yield dissimilar patterns of interactions with various dental materials or germanium prisms with high, medium and low critical surface tension [6, 18, 19]. The initial critical surface tension of a material was found to control the biological interaction potential of a substratum surface mainly through its influence on the mixed pellicle organisation [6, 18]. In tendency, less bacteria adhered to low-energy surfaces, indicating the relevance of low-energy surfaces for plaque control [19]. The application of amine fluorides reduces the surface free energy in vitro, and it was postulated that this may reduce bacterial colonisation [23].

Notwithstanding, even some in vitro studies found that bacterial adhesion is not affected by the colonised material covered by an experimental pellicle [125] or there are only minor effects of the different substrata [141].

On the first view, in vitro studies seem to be preferable as the experiments can be conducted under standardised and reproducible conditions [87], but a lot of studies have yielded clearly that in situ or in vivo gained pellicles differ significantly from in vitro pellicles [15, 76, 86, 116, 167,

168]. This affects not only the tenacity of the biofilm but also composition and enzymatic activities [40]. Intraoral maturation of the biofilm due to proteases and transglutaminases may account for these differences [12, 40, 47, 165, 166]. Accordingly, in situ or in vivo approaches are preferable despite high intra-individual and inter-individual variability and different modulating factors such as varying salivary flow, nutrition, bacteria in the oral cavity, soft tissues, etc. Sometimes, the biological variations are several times greater than the experimental errors [21, 42, 44, 45]. For studies on the influence of different substrata, in situ experiments with individual trays or splints for reproducible exposition of the samples in the oral cavity seem to be ideal. This proceeding allows investigation of the biofilm in the native state with a couple of methods [42, 45, 50].

## An easily accessible in vivo system for investigation of bioadhesion to different substrata—the oral cavity

Based on the in vitro data, considerable impact of different materials on bioadhesion in the oral cavity in vivo or in situ is conceivable. However, the supposed substrate-dependent effects are less pronounced under oral conditions.

Furthermore, protein adsorption as well as bacterial adherence are mainly determined by the surface roughness rather than by other material-specific physicochemical surface characteristics [110, 126].

### Physical surface characteristics

In vivo pellicle formation increased surface free energy of human enamel from 84 to 110 mJ/cm$^2$ within 5 min [22]. After 5 min, the surface free energy of pellicle-covered solids remained constant for at least 2 h [22]. In an in situ study with beagle dogs, surface free energies of different materials originally ranging from 22 to 134 mJ/m$^2$ converged to values between 60 and 100 mJ/m$^2$ after 30 min of oral exposure [148]. It was concluded that only the initial phase of protein adsorption during pellicle formation is influenced by the surface free energy [148, 149]. Nonetheless, the adhesive properties of the substrata may still be influenced by the certain material especially with regard to biofilm formation. In situ pellicle formation on artificial surfaces with different surface free energy featured a certain qualitative selectivity [6], but in situ exposition to the oral fluids compensated for the different surface free energies of solid substrata and reduced the water contact angles, respectively [14, 79, 89]. Thereby, the pellicle drastically increases the wettability of a vast number of materials and sealed of the effect of the original surface activity yielding a homogenizing effect [59, 79, 89, 149].

### Ultrastructure

The ultrastructure of the pellicle can be explored with several electron microscopic approaches such as scanning electron microscopy (SEM), TEM, atomic force microscopy (AFM) or field emission in-lens SEM [10, 11, 48]. Transmission electron microscopic studies show no clear ultrastructural differences of in situ formed pellicles on enamel, Vestopal and a couple of restorative materials [6, 10, 11, 48]. In a recent in situ study, AFM was used to monitor the protein adsorption on mica, silicon wafer and graphite. The surface free energy of the materials affected the rate of pellicle formation, whilst the overall size of the adsorbed protein aggregates appeared to be identical [51].

If compared with conventional electron microscopic studies, scanning force microscopic investigation has the advantage that no fixation of the samples is necessary. A scanning force microscopic study investigating the ultrastructure of in situ pellicles on a compomer and on enamel yielded completely different topographies of the pellicles after 60-min formation time [124]. The pellicle layer on the compomer was homogenous with a wavy substructure, whereas the enamel pellicle exposed a base layer on top of which larger proteins or protein agglomerates were adsorbed. Furthermore, the enamel pellicle had a substructure of a net of furrows. Despite the fact that cross-sections of pellicles on different surfaces as investigated in TEM yield a quite similar appearance [48], further research is necessary to visualise the surface morphology of in situ pellicles on different materials.

### Amino acid composition

Differing amino acid profiles were recorded for in vivo or in situ pellicles on enamel surfaces as compared with pellicles on dentures [27], plastic films [100] polyethylene terephthalate, restorative materials such as composite and amalgam [136, 138], primary teeth [135] or on fluoridated tooth surfaces, respectively [116, 118]. However, these differences manifested only as a slight shift of some amino acids such as glycine, isoleucine, serin, lysine and proline or the lack of cysteine and methionine, two amino acids present in the enamel pellicle in very small amounts near the limit of detection [27, 100, 116, 118, 136, 138]. Pellicles on primary teeth had an overall similar pattern as compared with permanent teeth, but the contents of serine, glycine and tyrosine were significantly different [135]. In general, the amino acid composition of the in vivo or in situ formed pellicles on different solid substrates was quite similar and correspond to other studies on the acquired enamel pellicle [117, 119]. A remarkable disadvantage of the amino acid analysis is that some amino acids may be hydrolysed during the preparation of the samples [40, 100,

117, 119]. Furthermore, no conclusion on the proteins or on the functionality of the pellicles can be drawn [40, 53, 70, 100]. Accordingly, quantification of typical pellicle proteins and glycoproteins offers additional information.

### Protein adsorption and composition

Protein adsorption during in vivo pellicle formation occurs selectively [27, 47, 56, 168]. This has been shown not only for enamel but also for denture base materials, titanium and nickel chromium alloy [27, 36, 64, 65, 102]. Many components of the acquired enamel pellicle were detected in in vivo or in situ pellicles on different restorative materials [27, 45], but some differences were observed. In contrast to enamel pellicles, cystatine, proline-rich proteins and low-molecular-weight mucin were not detectable in denture pellicles by sodium dodecyl sulphate polyacrylamide gel electrophoresis, whereas amylase, lysozyme sIgA, albumin and high-molecular-weight mucin were present in both [27]. Less albumin was detected on in situ titanium pellicles as compared with dental hard tissues [65]. Nevertheless, in a gold immunolabelling approach, the same amounts of amylase and lysozyme were detected on enamel, titanium and fieldspar ceramic [45].

### Enzyme activities

In contrast to other methods of protein analysis, evaluation of enzyme activity gives an insight in the biological function of the pellicle without desorption, denaturation or fixation of the initial oral biofilm. Enzymatic methods are usually based on photometric determination of the conversion rate of a certain substrate [36, 40, 42]. However, only few papers consider enzymes in the acquired pellicle on different materials [36, 42, 102], thereby some of them do not include a reference group [36], and others do not determine enzyme activities [65, 102]. In situ pellicles on denture base material yielded activities of all enzymes typically occurring in enamel pellicles [36]. Amylase and lysozyme are the most abundant enzymes in the acquired pellicle with high structural and functional significance [42, 45]. In situ experiments with six subjects yielded that the impact of dental hard tissues and different materials on the activity of amylase and lysozyme activity in the acquired pellicle is very limited [42].

## From pellicle to plaque—initial bacterial colonisation of different materials in vivo

The omnipresent in vivo formation of a pellicle in vivo reduces bacterial adhesion considerably irrespective of the underlying substratum and has a masking effect on materials' specific surface characteristics [63, 89, 126, 138]. In vivo and in situ studies on bacterial biofilm formation on dental materials are inconsistent but indicate a certain effect of different solid substrates on microbial colonisation [63, 92, 123, 126]. These effects are superimposed and overruled by the surface roughness of the materials [108–110]. Composition of plaque on different substrata featuring comparable roughness do not differ considerably [108]. At surface irregularities, protein agglomerates or microorganisms are protected against shear forces [110]. A systematic review showed that surface roughness above a $R_a$ threshold of 0.2 μm facilitates bacterial adhesion [143]. Accordingly, strong effects of solid substrates on biofilm formation and maturation were mainly observed with very pronounced differences of the surface structure [109]. Some in situ studies with different materials even yielded no ultrastructural differences of plaque on restorative materials and enamel as observed electron microscopically (compare Fig. 4) [10, 11, 50]. Another in vivo study gave clear evidence that the colonisation pattern of oral streptococci in 4-h-old in vivo plaque on plastic films is similar to that previously observed on natural tooth surfaces [113]. In an in situ approach including different implant materials, the number of adhering viable bacteria after 4 h of oral exposition depended on the surface properties, but after 48 h of plaque formation on the substrata, there were no differences detectable any longer [92]. The specimens had different roughness, contact angles and surface free energies, respectively [92]. In vivo plaque formation was evaluated for a number of other dental restorative materials such as amalgam, gold alloy, Cr–Co alloys, ceramic as well as for enamel and dentine [126]. Thereby, not only the amount but also the quality of the biofilm was monitored for 4 and 24 h, respectively [126]. The amount of early deposits on the different substrata depended on their surface roughness, whilst plaque formation after 24 h was qualitatively similar [126]. This lacking trend for preferential colonisation of common restorative materials corresponds to other in situ or in vivo studies [66, 71, 126, 129, 144]. Apparently, surface properties influenced only early bacterial adherence but not plaque maturation. Also, bacterial colonisation of adjacent restored proximal surfaces was investigated [63]. In general, restored proximal surfaces tend to be more highly colonised by *Streptococcus mutans* than sound surfaces, but gold is less colonised as compared with amalgam [63]. Adjacent gold surfaces are even less colonised than sound/sound proximal sites. In contrast, very high scores were recorded for neighbouring resin to resin surfaces [63]. Also, ceramic crowns seem to accumulate less plaque than adjacent normal tooth surfaces [98]. Nonetheless, even on polished gold cast restorations, considerable biofilm formation can be observed clinically after cessation of oral hygiene (Fig. 6). Overall, polymeric materials seem to

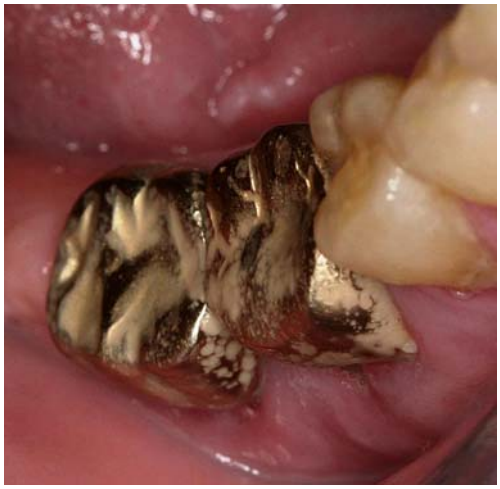

**Fig. 6** Biofilm formation on polished gold alloy surfaces after cessation of oral hygienic procedures for 8 weeks

accumulate the highest amounts of bacteria in vivo or in situ [63, 129–131, 138, 162]. In the oral cavity, hydrolysis of the silane interface between polymer matrix and anorganic filler particles as well as extensive wear of resin composite fillings and other degradation processes yield an increasing surface roughness of composite fillings. This aspect rather than physicochemical surface properties offers an explanation for the enhanced plaque accumulation especially on aged composite fillings [133, 134]. However, durability of modern composite materials is superior as compared with the materials used more than two decades ago [81]. In contrast, significantly less bacteria were detected on zirconium oxide discs as compared with titanium of similar roughness after 24 h exposition in vivo [123], and titanium nitride or zirconium nitride coatings reduced the number of adherent bacteria in vivo after 60-h exposure on implant materials [39]. Low-energy surfaces—even after several days of exposure in the human oral cavity—retained the smallest amount of adherent plaque due to the lower binding forces between bacteria and solid substrata [32, 107, 108, 161]. Such arrangements are determined by the initial surface energy status of the material. Low critical surface tension materials are unable to retain thick plaque deposits. At a critical thickness, presumably plaque layers peel away [18]. It should be mentioned that easy release of accumulated biomass will occur not at the lowest surface energies but slightly above between 20 and 30 mN/m, the so-called theta surfaces with an optimum between 22 and 24 mN/m. At this level, the adsorbed proteins seem to yield the lowest level of conformational changes or denaturation, respectively, required for tenacious binding to the surface [7].

The classical anti-adhesive surface coating Teflon (polytetrafluoroethylene, surface free energy 20 mJ/m$^2$) yields a less plaque accumulation in vivo and in situ as compared with other materials or enamel (60–80 mJ/m$^2$) [80, 107, 108, 161]. Furthermore, the accumulated bacterial biofilm can be removed more easily from Teflon-coated specimens, indicating a lower tenacity of the biofilm [80]. However, Teflon coatings do not have the physical characteristics needed for durable coating of solid surfaces in the oral cavity. New nanocomposites with a surface free energy of less than 20 mJ/m$^2$ having the mechanical properties of composite restorative materials are available for the coating of titanium or enamel [55]. These composites are based on polycondensation of methyl-triethoxy-silane, tetraoxy-silane and perfluoro-octyl-trimethoxy-silane with suspended $SiO_2$ nanoparticles [55]. The nanocomposite material exposes different components at the surface varying at the nano level. Adsorbing proteins are faced with different types of binding forces, decreasing the bond strength to the surface as compared with bare enamel [55]. At the moment, only heat curing material is available, but further studies are in progress to develop a light curing version [55]. However, the first in situ results are very promising for the establishment of an easy-to-clean material in modern dentistry. In an electron microscopic analysis of specimens exposed in situ, a strongly reduced adherence of bacteria was observed on the coated samples as compared with uncoated controls. Also, the 6-h in situ pellicle itself exposed a different ultrastructure. The 24-h bacterial biofilm on nano-coated enamel samples—if present—does not differ from that observed on uncoated samples, but with coated specimens, the detachment of bacterial layers from the basal layer of the pellicle was observed, indicating self-cleaning effects (Fig. 5) [55]. Intraorally active shearing forces are strong enough to remove the outer pellicle layer and adhering bacteria from the nanocomposite coating. The nanocomposite does not work by inhibiting the adhesion of the proteins but by reducing adhesion strength or tenacity, respectively. The tenacious basal layer which does not detach from the surface might ensure the lubricating effect of the pellicle to prevent enhanced tooth wear [60]. Also, typical enzymes such as lysozyme or peroxidase are present in the in situ pellicle on the nano-coated enamel samples in an active conformation, indicating that the protective properties of this proteinaceous layer are maintained (unpublished data).

In accordance with these actual data, in a previously performed in vivo study by Olsson et al. [98], ceramic crowns with a highly hydrophobic surface coating accumulated almost no plaque, indicating a biofilm formation of low shear stress resistance.

## The pellicle, a physiological masking of different surface properties?

The reviewed in vivo or in situ data on the proteinaceous pellicle and on the bacterial biofilm on different substrata

are somewhat bewildering. Pellicles on different substrata have a certain uniformity. This was shown for enzyme activities as well as for the ultrastructure or for the general amino acid composition, respectively [11, 42, 45, 48, 100, 136, 138]. Observed differences were not very pronounced and are often attributed to selective mechanisms of protein adsorption during the very initial stages of pellicle formation [35, 56, 100, 149]. During the deposition of the pellicle layers, the specificity of adsorption process onto substrata with different physical properties is assumed to be lost progressively [100]. Salivary protein adsorption tends to level out the original differences in substratum surface free energy [148, 149] and evens the surface relief [126]. Thereby, the micelle-like globular structures and the heterotype complexes composed of salivary proteins and mucins provide fast filling or sealing of surface irregularities or rough structures, respectively [53, 112, 120, 157, 170].

However, bacterial adhesion and biofilm formation were often shown to follow the pattern expected on the basis of critical surface tension and of the original surface free energy of a material together with the shear forces present [107, 108, 110, 129–131, 149, 162]. The differences in bacterial colonisation of dental materials are not fully explored. There are only postulates for the phenomenon of different bacterial colonisation despite the rather homogenous pellicle layer. It is likely that a mechanism exists by which surface characteristics of a solid are transmitted through the adsorbed film (Fig. 1) [18, 95, 103, 146]. The range of the forces responsible for adherence of proteins and bacteria seems to be of considerable significance for this observation [94, 95, 106, 143].

It may be postulated that long-range forces act through the pellicle layer and impact at least the first approach of the microorganisms [18]. Further on, despite the described homogenising and masking effects of the pellicle formation [59], there may be conformational differences of the adsorbed proteins on different materials [106]. This is especially relevant for the bacterial receptors or for the active sites of enzymes [2, 37, 40, 106]. Also, rearrangement and desorption of external parts of the pellicle layer are assumed to be a key to different bacterial colonisation of dental materials [95]. Despite the fact that the pellicle on different surfaces exposes the same enzyme activities at the surface and despite the ultrastructural homogeneity [42, 48], the pellicles may be of different tenacity, as indicated by measurement of the adhesion forces [124]. The tenacity of the adsorbed proteins is a result of surface properties such as wettability, electric charge, surface free energy, etc. [95]. Pellicles of different tenacity detach or peel off in a different manner or yield a different turnover, respectively [6, 156]. The bacterial biofilm and the outer layer of the pellicle detach or shed of from ceramic surfaces, leaving only the electron dense basal layer (Figs. 4d and 5). This phenomenon was observed only with polished or glazed ceramic surfaces and especially on new low-energy nanocomposite surfaces [55]. Salivary films are adsorbed on low-energy surfaces with a loose, more native configuration, and therefore, they could be more easily desorbed [6, 156]. In conclusion, physico-chemical surface properties are only in part counterbalanced by pellicle formation. Conformational differences of the adsorbed proteins induced by short-range forces as well as long-range forces transmitted through the pellicle layer may account for this phenomenon [18, 37, 95, 103]. Nevertheless, pellicle formation on all orally exposed surfaces ensures the ubiquitous biological function of this proteinaceous film as a lubricant [53].

Despite the nature of the pellicle to mask the physical surface properties of different solid substrates, surface-determined long-range interactions influence the bacterial colonisation of the pellicle layer [143]. This allows the establishment of low-energy surfaces with low bacterial colonisation without eliminating the protective and lubricating pellicle layer.

All in all, extensive knowledge and exploration of the in situ pellicle is the fundament for biofilm management strategies in the oral cavity. Despite extensive investigation of the amino acid composition and the ultrastructure, some aspects of pellicle research are still in the beginning. Many small peptides were not characterised until now [53, 70, 128]. Furthermore, the structural and conformational changes of salivary proteins due to the process of adsorption are of high importance to understand bioadhesion on the molecular level. [37, 106] Thereby, the oral cavity offers the unique opportunity to carry out in situ studies in noninvasive approaches [42, 45]. Valuable information of general scientific interest on bioadhesion processes in vivo can be gained from dental research using the oral cavity as a model. In this context, it has to be pointed out again clearly that in situ and in vitro studies yielded differing results, indicating the limitations of in vitro approaches [15, 40, 167].

## Conclusions

- In vitro studies do not reflect bioadhesion in vivo. Accordingly, in situ or in vivo studies are strongly recommendable in order to understand the details of bioadhesion in man.
- The oral cavity is an excellent in vivo model for the investigation of protein–surface interactions noninvasively.
- Pellicle formation masks the physicochemical surface properties of dental materials to a certain extent.

However, bacterial adhesion and tenacity of the bacterial biofilm is considerably influenced by long-range forces transferred through the pellicle layer.

– Low-energy surfaces are desirable for biofilm management in the oral cavity; the realisation of such materials for clinical use is conceivable.
– Besides chemical and physicochemical surface characteristics of dental materials, the surface roughness is of great relevance for bioadhesion in the oral cavity.

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