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The mucosal pellicle - an underestimated factor in oral physiology

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Abstract

Bioadhesion and bio-adsorption of proteins, glycoproteins and other biomolecules are ubiquitous phenomena in the oral cavity. While the protective role of the adsorbed salivary biomolecules on teeth (the acquired enamel pellicle) is well established, it has yet to be defined whether comparable processes occur on the desquamating oral soft tissues. The general term for these layers is pellicle, but due to the different characteristics of the coated surfaces the enamel pellicle and mucosal pellicle are their own entities. There is considerable information on the enamel pellicle, whereas only limited data are available on the mucosal pellicle. This can be attributed to the difficult standardized preparation of this biological structure. Based on the present knowledge the abundant and characteristic components of the mucosal pellicle include secreted soluble mucins (MUC5B, MUC7), membrane-associated epithelial mucins (MUC1), and to a lesser degree CA VI, sIgA, and cystatin. However, it seems to be of completely different ultrastructure as compared with the enamel pellicle. Since it is comprised of larger glycoproteins retaining water, it might be considered as a hydrogel, and it appears to have a lower tenacity than the enamel pellicle. Maturation and turnover are influenced by the delivery of salivary proteins, by the flow of saliva and the underlying desquamating oral epithelium. Its probable functions include lubrication and moisture retention.

In general, the mucosal pellicle can be regarded as an underestimated key player in oral physiology.

Introduction:

Many important physiological processes, such as tasting, eating and chewing food, take place in the oral cavity; saliva is critical to all these functions. Furthermore, saliva provides several protective components to preserve the healthy integrity of the oral tissues (Carpenter, 2013; Dawes et al., 2015; Sreebny, 2000; Villa et al., 2015). How saliva performs all these different functions depends to a large extent on its interactions with the surfaces of the mouth (Amerongen & Veerman, 2002; C. Hannig & Hannig, 2009; M. Hannig & Joiner, 2006). Generally, interfaces between liquid and solid tissue are prone to bioadhesion processes. In the oral cavity, tissues can be classified as either soft or hard surfaces which have particular qualities that determine their interaction with saliva and salivary biomolecules. The adsorption of specific salivary biomolecules and proteins is to some extent determined by the physicochemical properties of the substratum such as surface polarity, wettability and the expression of potential receptors (see figure 1). Electrostatic, hydrophobic, and van-der-Waals interactions as well as covalent bonds generally induce the spontaneous adhesion processes which are supposedly driven by a gain in entropy (C. Hannig & Hannig, 2009; M. Hannig & Joiner, 2006; Norde, 1986). Additional protein interactions and intermolecular cross-linkage are suggested to contribute to the formation of a more complex layer, the pellicle, which is initially free of bacteria (C. Hannig et al., 2007; Lendenmann, Grogan, & Oppenheim, 2000). Teeth are the main hard surfaces in the mouth (calculus, dental restorative materials and prostheses being others) and the role of the acquired enamel pellicle in protecting the teeth from erosion and abrasion is well known and understood (C. Hannig & Hannig, 2009; M. Hannig & Hannig, 2014). The acquired enamel pellicle is a specific subset of salivary biomolecules that strongly bind to the ionic surface of the teeth to maintain a high concentration of calcium directly around the tooth as well as providing a buffering, and lubricating layer of salivary protein. Furthermore, the pellicle on solid substrates exposed to the oral surfaces seems to be of high uniformity masking the physicochemical characteristics to a considerable extent (C. Hannig & Hannig, 2009; M. Hannig, 1997, 1999a). Whereas the mucosal pellicle is also a subset of salivary proteins, it is quite different from the acquired enamel pellicle (figure 1 a, b). Recent studies suggest that it is composed mostly of large molecular weight glycoproteins such as the salivary mucins MUC5B and MUC7 as well as secretory IgA (Gibbins, Proctor, Yakubov, Wilson, & Carpenter,

2014). Electron microscopic studies indicate that the mucosal pellicle (Morzel, Siying, Brignot, & Lherminier, 2014) has a different structure from that found on teeth (M. Hannig, 1999b).

However, the mucosal pellicle also serves as a protective layer ensuring retention of moisture and lubrication of the oral epithelia as well as protection against excessive bacterial colonization (Bradway, Bergey, Jones, & Levine, 1989; Ployon et al., 2016). The present review paper aims to give an overview on present knowledge of the mucosal pellicle and to set out hypotheses and open questions for further research.

Terminology:

It may be wise at this point to define correctly the terminology as the literature is variable; sometimes because of the scientific field of the journal. For example, in the physical (exact) sciences the mucosal pellicle, and indeed the enamel pellicle, is often referred to as the (salivary) conditioning film (Macakova, Yakubov, Plunkett, & Stokes, 2010) often from a microbiological point of view (Veeregowda et al., 2013). The term conditioning film is rather misleading, since it suggests that the only function of the pellicle layer is to facilitate subsequent binding, such as bacterial colonization. However, this completely neglects the physiological and protective properties of this proteinaceous layer, which will be considered later. In medical/ immunological journals the mucosal pellicle has recently been described by the term “oral mucosal barrier complex”, rather than mucosal pellicle (Asikainen et al., 2012). In microbiological journals the term salivary pellicle also occurs (Nobbs, Vickerman, & Jenkinson, 2010) but since there is also a dental or enamel pellicle, which is quite different, this term seems inadequate. In dental related journals the mucosal pellicle is not often distinguished from the salivary film or residual saliva that coats the surfaces of the mouth. Saliva forms a thin film in the mouth (Dawes, Watanabe, Biglow-Lecomte, & Dibdin, 1989) termed the salivary film which is different in protein composition from whole mouth saliva expectorated from the mouth (Pramanik, Osailan, Challacombe, Urquhart, & Proctor, 2010). The salivary film’s protein composition varies throughout the mouth and adjacent to ductal opening from salivary glands. It often resembles that of ductal saliva. Thus on the inside cheek, near the opening of the parotid duct (Stenson’s duct) many parotid salivary proteins (such as basic proline-rich proteins) are apparent although not causing exclusion of other

proteins such as mucins, which are not released by the parotid gland. Accordingly, there appears to be some mixing of salivas from different glands probably reflecting tongue activity but there is also some heterogeneity.

In trying to define the term “mucosal pellicle”, it might be helpful here to consider the degree of attachment of salivary proteins and other components within our definition of what should be considered as part of the mucosal pellicle. For the enamel pellicle there has been much debate over the definitive pellicle components as it was difficult to completely remove it from the teeth. Usually polishing is used to establish a new surface which in itself changes the surface roughness and physical properties (surface tension, associated ions etc.). On the mucosa the basic surface is constantly being replaced which also complicates the timing of formation of a pellicle. In a recent study, it was sought to establish how strongly salivary proteins were attached to the sloughed epithelial cells (Gibbins, Proctor, Yakubov, Wilson, & Carpenter, 2014). Cells were washed with different solutions (water, tris-buffered saline and SDS) and then homogenized and loaded onto gels, and immunoblotted to assess which salivary proteins were still present. Many proteins including statherin, cystatins and carbonic anhydrase 6 were rapidly washed off. Only the mucins MUC5B and MUC7 and sIgA remained intact following three washes. However, this study has several limitations. Firstly, it was targeted and a proteomic approach might offer information on the complete list of proteins. Secondly, there is an assumption that sloughed epithelial cells are the same as cells still attached to the mucosa. Thirdly, the strength of the washes was limited by the integrity of the cells, i.e. any stronger solution than 0.5 % SDS caused the cells to lyse prematurely. In practice it might be suitable to define adsorbed proteins and glycoproteins that cannot be removed by a water jet as a pellicle layer. The degree of attachment might be further re-inforced by the action of secreted and membrane-bound transglutaminases crosslinking proteins together into a more mature pellicle, as occurs for the enamel pellicle (C. Hannig, Spitzmüller, Miller, Hellwig, & Hannig, 2008). Early pioneering studies certainly suggested a role for transglutaminases (Bradway et al., 1989). Another complication is that the pellicle never forms in a sterile environment. Oral bacteria and other microflora will also be present in the mouth, which may affect formation or degradation of the pellicle. A pure pellicle, before formation of a three-dimensionally organized bacterial biofilm, will always contain randomly adherent bacteria (see figure 2 and

3). However, for the purposes of future terminology, it might be suggested that oral bacteria and other exogenous components should not be considered a key feature of the mucosal pellicle. They may well bind to it with some considerable attraction but they are not vital to its formation, unlike the salivary proteins (C. Hannig et al., 2007).

By rejecting the term “salivary pellicle” because it is too vague (see above), we also reject the term “salivary mucosal pellicle” because although the major components do come from saliva, they interact with epithelial surface membrane mucins and these must be included as key components. Thus, the pellicle will not be exclusively of salivary protein origin. Indeed the use of proteomics may reveal components that come from the epithelial cells that hitherto have not yet been identified. For these reasons this review has settled on “mucosal pellicle” as being the term of preference for the adsorbed protein layer on oral epithelial cells of the mouth.

Thus, for the mucosa it might be concluded that the definition of the mucosal pellicle covers those components attached to the cell surface that cannot be easily removed by water or other low ionic washes. On top, there will be the rather motile salivary film; a thin layer of liquid containing proteins, bacteria, food components and ions that delivers and replenishes the pellicle beneath it (figure 1 b). In the next section we will consider the formation of the pellicle by first reviewing the formation of the enamel pellicle, of which we know much more.

The enamel pellicle - a benchmark in research on bioadhesion in the oral cavity

The topic of the present review is to evaluate current knowledge on the mucosal pellicle; however, pellicle formation on the teeth should be described in brief as some kind of a reference or benchmark. Bio-adsorption on solid surfaces in the oral cavity can be evaluated *in situ*, *in vivo* and *in vitro*. There are many *in vitro* studies on pellicle formation based on incubation of material samples with collected saliva (C. Hannig & Hannig, 2009). However, this does not mirror the situation in the oral cavity and *in situ* or *in vivo* approaches are to be

preferred. For *in vivo* experiments, the pellicle is scraped off from the dental hard tissue with curettes or wiped down with small sponges. However, these approaches do not ensure complete removal of the basal pellicle layer. Due to these drawbacks, *in situ* studies are preferable. Enamel samples or other solid substrates are exposed to the oral fluids with the aid of splints or trays, respectively (C. Hannig & Hannig, 2009). After removal from the oral cavity, the samples can be analyzed with different electron-microscopic techniques, with enzyme-assays and with modern metabolomic, lipidomic and proteomic methods (C. Hannig, Spitzmüller, & Hannig, 2009; M. Hannig, 1999a, 1999b; Lee et al., 2013; Reich, Kümmerer, Al-Ahmad, & Hannig, 2013; Zimmerman et al., 2013)

Accordingly, despite the low thickness of the pellicle, there are numerous studies on the enamel pellicle. Based on some ambitious research over the last years, fundamental knowledge has been gathered covering the morphology, composition and function of the acquired enamel pellicle (C. Hannig, Berndt, Hoth-Hannig, & Hannig, 2009; C. Hannig, Ruggeri, et al., 2008; M. Hannig & Balz, 2001; Lee et al., 2013; Zimmerman et al., 2013). Referring to broad transmission electron microscopic analysis, the pellicle's thickness and ultrastructure depend on the formation time and on the localization in the oral cavity. However, due to some universal bioadhesion processes at the ionized tooth surface, a characteristic two-phase basic structure, consisting of an initially formed electron-dense basal layer covered by globular structures, can be detected in all sites of the oral cavity (M. Hannig & Balz, 1999, 2001; M. Hannig & Joiner, 2006; M. Hannig, Khanafer, Hoth-Hannig, Al-Marrawi, & Acil, 2005) (figure 1 a).

Initial protein adsorption on the mainly inorganic and acellular structures is governed by physicochemical interactions such as van der Waal's forces, dipole-dipole effects and hydrophobic interactions (M. Hannig & Joiner, 2006). Only the following steps of pellicle formation are characterized by different kinds of bonds between organic molecules such as proteins and glycoproteins. This represents one essential difference as compared with the mucosal pellicle.

Recent proteomic analyses have identified up to 130 different peptides and proteins in the pellicle after 120 min of intraoral formation (Lee et al., 2013). Characteristic proteins are statherin, histatins, proline-rich-proteins, lactoferrin and cystatins. Serum proteins are also present with albumin making up a large proportion. Although not being as dominant as in

the mucosal pellicle, MUC5B as well as MUC7 have also been identified in the matured acquired enamel pellicle which is suggested to be due to protein-protein interactions and their affinity to bind other pellicle proteins such as α -amylase (Lee et al., 2013)(table 1). Also sIgA is a characteristic component of the enamel pellicle (Deimling et al., 2007; M. Hannig & Joiner, 2006). One important focus of previous research was the analysis of several enzymes incorporated in the pellicle in an active conformation. Lysozyme, amylase and peroxidase are the most abundant human enzymes in the enamel pellicle accompanied by the bacterial glycosyltransferases (C. Hannig, Hannig, & Attin, 2005; C. Hannig, Ruggeri, et al., 2008; Kirsch et al., 2017). Of course at different concentrations, they are all detectable in the early stages of pellicle formation which contributes to the protective as well as pathophysiological properties of the pellicle regarding bacterial adhesion.

Fast pellicle formation is ensured by the adsorption of micelle-like structures, supramolecular pellicle precursors (Soares et al., 2004) or heterotypic complexes, respectively. They have a composition that is comparable with the initial enamel pellicle. Characteristic components are amylase, lysozyme, proline-rich proteins, histatins and others (Vitkov, Hannig, Nekrashevych, & Krautgartner, 2004). It has not been investigated until now whether these structures are also involved in pellicle formation on the mucosa though salivary proteins are mainly secreted in this form.

Research on the mucosal pellicle - methodical approaches, challenges and problems

Although it is very tempting to derive further knowledge about the mucosal pellicle from the partially conceived bioadhesion processes at the tooth surface, the unmistakable particularities of the soft oral tissue need to be emphasized. Regarding the oral mucosa, adhesion on the molecular level is not only confronted with the broad presence of dynamic processes due to food- and substance intake, salivary clearance and abrasive influences but is additionally challenged by the mobility of the surface itself and the regular desquamation of the tissue. The mineralized non-shedding immobile tooth surface enables a variety of short- and long-range intermolecular forces between the surface ions and the surrounding molecules. As the interface with the soft oral surfaces is cellular, it can be assumed that the

outer layer of mucosal cells provides additional morphological adaptive mechanisms or selective receptors, respectively, likewise to allow attractive as well as repulsive interactions with the salivary biomolecules (Asikainen, Mikkonen, Ruotsalainen, Koistinen, & Kullaa, 2014; Gibbins, Yakubov, Proctor, Wilson, & Carpenter, 2014). In fact, scanning and transmission electron microscopic analyses have shown the apical cell membrane forming the interface to the oral cavity has continuous membrane folds, termed microplacae (Asikainen et al., 2014). Therefore, when studying the oral mucosal pellicle one major challenge is determining a suitable mimetic for the oral mucosal surface. Naturally one might consider the best substrate would be oral epithelial cells and indeed several studies have used these. However they have several problems. Sloughed cells found in saliva have, by default, already been coated in saliva and formed, to varying degrees, a mucosal pellicle (figure 2, 3). Thus they are not suitable for dynamic studies of formation. Even well washed sloughed cells will not bind further salivary proteins (unpublished observation). Secondly the sloughed epithelial cells do not readily attach to plastic culture dishes and are therefore not capable of longer term studies. Interesting results on the ultrastructure of the mucosal surface and the adherent pellicle were gained from biopsies. However, the amount of biopsy material is limited and the samples only suitable for certain methods (Vitkov, Hannig, Krautgartner, & Fuchs, 2002; Vitkov, Krautgartner, Hannig, & Fuchs, 2001; Vitkov, Krautgartner, Hannig, Weitgasser, & Stoiber, 2002). Brushing biopsies also have certain drawbacks. The process of brushing might alter the ultrastructure of the cells and the adsorbed layers of different tenacity. It would therefore be desirable to have an oral mucosal mimetic for *in situ* studies.

Several studies have used either glass or plastic as a mimetic particularly in the microbial field of studies. Often these studies consider the oral mucosal pellicle as being an important factor in bacterial and fungal binding to oral surfaces (Nobbs et al., 2010) but then do not consider that the subset of binding proteins will depend on the substrate. To illustrate this, a recent study used beads with different surface chemistries to examine the likely forces driving the interaction (Gibbins, Yakubov, et al., 2014). It was demonstrated that the subset of salivary proteins binding varied according to surface charges implicating electrostatic interactions as being important. Crucially though it was shown that these surfaces were very poor binders of salivary mucins and, thus, not a good mimic of the *in vivo* mucosal pellicle. Generally, it should be kept in mind that these solid surfaces cannot really mimic the

biological and physicochemical surface characteristics of epithelial cells (compare figure 1 a and b). Accordingly, the likely receptors on the oral epithelial cells were reconsidered. The surface glycoproteins have been well characterized in the past (Hori, Sugiyama, Soma, & Nishida, 2007; Nobbs et al., 2010) as well as the mucins 1, 4 and 16 which would be first layer of interaction due to their length. Thus it was considered whether membrane-bound mucins helped to mediate salivary mucin binding. CHO (Chinese hamster ovary) cells expressing MUC1 have provided clear evidence that MUC7 binding is governed by MUC1 expression. However, MUC5B binding to these epithelial cells was still observed even when MUC1 was absent (unpublished data). This suggests that other surface mucins such as MUC4 and 16 may also play a role for MUC5B binding although these have been largely unstudied so far.

The use of immortalized oral epithelial cells has been adopted with success to show salivary mucin binding (Morzel et al., 2014) in a single layer format. But these cells (often TR146 or OKF6/TERT2) can be grown as multilayers, and so replicate a mucosal section (Dongari-Bagtzoglou & Kashleva, 2006; Ployon et al., 2016). These 3D cultures have been widely used for bacterial/ fungal interactions where there have been clear differences compared with 2D cultures (Pinnock, Murdoch, Moharamzadeh, Whawell, & Douglas, 2014). A recent study adopted a stable TR 146 cell line which had been modified by transfection in order to express MUC1. This allowed evaluation of the interactions between membrane-associated MUC1 and salivary MUC5B (Ployon et al., 2016). However, a drawback of cell culture based experiments is that no dead cells are present at the surface which is typical for the *in vivo* situation (Ployon et al., 2016; Squier, 1991). One problem with trying to incubate cell lines with saliva is the higher hypotonicity of saliva compared with cell culture medium. So the method most often used is to dilute the saliva in the culture medium which works well and prevents cells lysing due to osmotic pressure but reduces the likely interactions that may be present in the mouth.

Clearly an *in situ* mimetic of mucosa cannot be cell-based. The risk of infection would be too high to allow most researchers to pursue this line of enquiry. Only autologous cells would offer an opportunity. Instead different materials have been used to mimic the mucosa. In studying the tribology of saliva, Selway and Stokes have used a PDMS (Polydimethylsiloxane) substrate prepared in such a way as to give a deformable, soft

structure (Selway & Stokes, 2014). These experiments provided novel and interesting data on the lubricating capacity of saliva on a deformable surface. Although the composition of salivary proteins binding was not reported, other studies have found PDMS relates favorably to pig's tongue, a commonly used substitute for human oral mucosa (Ranc et al., 2006). Further studies are required to confirm the presence of salivary proteins to determine how well PDMS mimics the oral mucosa in forming the oral mucosal pellicle. Potentially also other hydrophilic materials with a high water content such as hydrogels might serve as a substrate for *in situ* experiments mimicking the outer surface of mucosal cells. Last but not least epithelial cells adsorbed to the enamel pellicle and detected as secondary finding during fluorescence microscopic and electron microscopic imaging of the dental *in situ* pellicle offer interesting information on the interactions of oral biomolecules and epithelial cells (figure 2, 4).

Different types of oral mucosa

Besides finding the right method to acquire adequate samples, different types of oral mucosa have to be considered. The mucosal pellicle might differ considerably according to localization in the oral cavity. Also the level of keratinization, the histology and the roughness as well as the site-specific dynamics of the oral fluids will obviously have an impact on this structure. Typical examples for the different types of soft tissue surfaces are palatal mucosa, attached gingiva, buccal mucosa, tongue and taste buds, lip as well as interdental papilla.

Mucosal cells have a dynamic cell membrane mainly composed of phospholipids with numerous receptor proteins, channel proteins and transporter systems (fluid mosaic). Ions and biomolecules released by the cells as well as membrane enzymes have a considerable impact on the process of bioadsorption (figure 1b). It has to be kept in mind that the cells are coated by the glycocalyx (Bradway et al., 1989). This polysaccharide matrix is composed of glycolipids, glycoproteins and proteoglycans. Last but not least, the mucosa and its level of keratinization are of high variability (Watanabe et al., 2013). There are soft and smooth, non-keratinized surfaces like the buccal sites as well as rugged and keratinized structures such as the tongue or the palatal mucosa, respectively (Bradway et al., 1989; Gibbins,

Yakubov, et al., 2014). The structure of microplacae is mimicked in recent cell culture models (Ployon et al., 2016). It has to be kept in mind that dead cells are present at the shedding surfaces of the mucosal cells due to turnover processes (figure 2, 3). In terms of micromorphological varieties, different types of microplacae as well as their uneven density have been described (Asikainen et al., 2012). It has been observed that the cells of the buccal and lip mucosa form primarily branched microplacae, while they were “parallel” in the cells of the tongue and in the floor of the mouth. The surface ultrastructure of the keratinized epithelia had a notably pitted appearance. Although the involvement of the microplacae in bioadhesion processes has not yet been clarified completely, it must be suggested that they contribute to the attachment of salivary components at the cell surface. All these characteristics represent a great heterogeneity in mucosal pellicles, which is further exacerbated by the generally high turnover rate of the oral soft tissues with a big impact on the process of bio-adsorption. The turnover rate of the superficial part of the oral mucosa was calculated to be 2.7 h (Dawes, 2003; Ployon et al., 2016).

Ultrastructure of the mucosal pellicle

The group around Morzel *et al.* has established an interesting approach to visualize the mucosal pellicle in a defined manner. Gold-immuno-labelling of a typical pellicle component (MUC5B) was performed and allowed defined evaluation of the mucosal pellicle (Morzel et al., 2014). The findings of this study fit well to other electron microscopic studies on this topic.

The mucosal pellicle's ultrastructure coating the characteristic epithelial microplacae (Asikainen et al., 2012; Kullaa, Asikainen, Herrala, Ukkonen, & Mikkonen, 2014) seems to be of low density as compared with the enamel pellicle and is rather discontinuous (figure 4a). The thickness varies and reaches up to 100 nm (Morzel et al., 2014). In contrast to the enamel pellicle, no electron-dense basal layer can be observed or it is at least not distinguishable from the cell membrane (figure 4) (Asikainen et al., 2012; Kullaa et al., 2014; Morzel et al., 2014; Vitkov et al., 2001). The pellicle-like structures are scattered and rather heterogeneous (Vitkov et al., 2001) (figure 4). The ultrastructure is partially filamentous and

sometimes of a fine granular appearance (Watanabe et al., 2013). In line with these observations the ultrastructure of an experimental pellicle formed on TR 146/MUC1 cell culture yielded a loose filamentous network covering the microplicae (Ployon et al., 2016). Watanabe et al. (2013) investigated the ultrastructure of the rat tongue mucosal cells and adherent bacteria. In most cases bacteria associated with the epithelial cell membrane were surrounded by glycoproteins. However, it is not clear whether these glycoproteins are of bacterial, cellular or salivary origin (Watanabe et al., 2013).

In contrast to dental enamel where the pellicle is a prerequisite for any bacterial colonization, direct attachment of bacteria to epithelial cells is possible and has been observed in the oral cavity (Chagnot, Zorgani, Astruc, & Desvaux, 2013; C. Hannig & Hannig, 2009; Vitkov, Hannig, et al., 2002; Vitkov et al., 2001; Vitkov, Krautgartner, et al., 2002). However, TEM-images of epithelial cells adsorbed onto enamel surfaces *in situ* indicate that pellicle structures can also mediate the interactions between bacteria and epithelial cells (figure 4).

Composition:

Based on the present knowledge, the mucosal pellicle seems to be mostly composed of the salivary mucins MUC5B and MUC7 (see table 1). In a complex with these mucins is secretory IgA which becomes concentrated to a much higher level than that found in saliva. Earlier studies using *in vitro* methods found some other salivary proteins in the mucosal pellicle including cystatin and some proline-rich proteins (Bradway et al., 1989; Yakubov, Macakova, Wilson, Windust, & Stokes, 2015) - a finding that was not confirmed in more recent experiments (Asikainen et al., 2012; Gibbins, Proctor, Yakubov, Wilson, & Carpenter, 2014; Kullaa et al., 2014; Morzel et al., 2014). Although not as tightly bound as mucins, other proteins, such as cystatin and PRPs, are likely to contribute to the boundary lubrication, hydration and microbial interactions of the mucosa as indicated by *in vitro* experiments (Yakubov et al., 2015).

Unlike the enamel pellicle in which statherin, histatin, acidic proline-rich proteins, amylase and lysozyme are the main components, the main components of the mucosal pellicle are the salivary mucins and secretory IgA. The specificity of the adsorption process is best illustrated by amylase. Despite amylase being the most abundant single protein in saliva, less than 1% bind to the mucosal pellicle (Gibbins, Proctor, Yakubov, Wilson, & Carpenter, 2013). This demonstrates that the mucosal pellicle, like the enamel pellicle, is mediated by specific interactions and is not merely residual saliva.

Saliva is a mucin-containing solution that coats the oral epithelium with a mucus layer. The mucus layer can be considered a hydrogel - it is a protein and glycoprotein backbone retaining high volumes of water. This provides the properties necessary to lubricate and protect the underlying mucosa (Klein, 2012). In most other epithelial surfaces the process of mucin deposition is simple. Abundant mucin secreting cells line the mucosa and secrete high concentrations of mucin directly onto the surface; renewing the layer from beneath (Verdugo, 2012). However, the mouth as an open system is different from other mucosal surfaces. Over 90% of saliva is produced by the major salivary glands, connected to the mouth via ducts and the saliva is delivered on top of the mucosa. Furthermore, major glandular saliva has a much lower concentration of mucin than that produced by minor salivary glands or goblet cells in the oesophagus, for example. Thus the process by which

mucins accumulate at oral surfaces to form mucus is unclear (Bradway et al., 1989; Pramanik et al., 2010).

Currently, there is little information on the consequences of a deficient mucosal pellicle. Patients suffering from a dry mouth are likely to have a defective mucosal pellicle (since saliva is required to form the pellicle) and they have significantly diminished quality of life due to increased difficulty in talking, eating and swallowing of foods. This applies especially for patients after radiation of head and neck or patients suffering from Sjögren's syndrome (Rogers et al., 2016; Tanasiewicz, Hildebrandt, & Obersztyn, 2016; Vissink, Spijkervet, & Van Nieuw Amerongen, 1996). In addition, these subjects have greatly increased rates of dental caries and increased number of oral infections (mostly oral thrush caused by *Candida albicans*). Although artificial salivas do exist, they perform poorly compared with saliva since they do not provide the same proteins or physical properties of saliva.

Around 90% of all saliva is secreted by the major salivary glands; parotid, submandibular and sublingual. Mucins are not produced by the parotid gland which is the largest gland in humans. However hundreds of minor glands line the oral mucosa which produce a mucin-rich saliva. It might then be argued that the minor glands are responsible for the mucus layer on the oral mucosa. However, mucin-secreting glands are not detectable on the tongue where a mucus layer is still present (Pramanik et al., 2010). The varying thickness and composition of the salivary film on the oral mucosa has been assessed using filter strips to collect samples. Electrophoretic analysis revealed the thickest film was present on the tongue where no mucin-secreting minor glands exist and the thinnest on the hard palate (Pramanik et al., 2010) where many palatal minor glands lie. This demonstrates that there is some movement of mucin molecules around the mouth.

Compared with minor saliva, whole mouth saliva has a much lower mucin concentration (approx. 50%, see figure 1) and, thus, requires a mechanism to concentrate it at mucosal surfaces to provide a lubricating layer. This process does not happen automatically, and concentrating mucin onto epithelial surfaces is not straightforward. Factors that might be important are the physical properties of the mucins. During synthesis by secretory cells the mucin molecules are packed into secretory granules within cells. To shield the abundant negative charges on the molecules calcium is also present (Kesimer, Makhov, Griffith, Verdugo, & Sheehan, 2010). Upon secretion from the cell the mucin unpacks into elongated

highly hydrated structures probably by the chelation of the calcium. One theory suggests that bicarbonate ions play a role in the chelation of calcium and the unpacking of mucin (Quinton, 2010). This has been evaluated in saliva by examining the extensional rheology of saliva in the presence of added bicarbonate (Vijay, Inui, Dodds, Proctor, & Carpenter, 2015). Saliva has excellent extensional properties and readily forms strings of saliva (phenomenon termed spinnbarkeit) which drain into a beads-on-a-string type morphology (Bhat et al., 2010). This property of saliva is relatively short-lived (approx. 30 min after salivary collection) and can be attenuated by adding bicarbonate ions. Maybe these mechanisms and interactions contribute also to surface interactions and thereby to the formation of the mucosal pellicle.

In contrast to the enamel pellicle, not only physicochemical interactions, especially hydrophobic interactions, but also covalent bindings contribute considerably to formation of the initial mucosal pellicle (Gibbins, Proctor, Yakubov, Wilson, & Carpenter, 2015; Ployon et al., 2016).

Mucin-mucin interactions are likely to be an important factor for binding. Oral epithelial cells are known to express a membrane-bound mucin MUC1 and, thus, interactions between MUC1 and salivary mucins may lead to the development of the mucosal pellicle (Ployon et al., 2016). Our initial studies have shown that a MUC1 expressing cell line (CHO cells, gift of Prof Hughey, Univ Pittsburgh, USA) showed increased binding compared with the same cells without MUC1 expression (in part unpublished data) (Gibbins et al., 2015). In addition, using a cell line that secretes mucins (HT29 treated with MTX) increased the binding of salivary mucins (Gibbins et al., 2015). Hydrophobic interactions are also considered relevant driving forces for formation of the mucosal pellicle (Gibbins et al., 2013; Gibbins & Carpenter, 2013; Gibbins, Yakubov, et al., 2014), and epithelial transglutaminase is regarded as a relevant enzyme for the crosslinking of membrane proteins and salivary components (Bradway et al., 1989; Bradway et al., 1992; Gibbins, Yakubov, et al., 2014).

Discussion

The review of the present publications on the mucosal pellicle indicated that there is only limited knowledge on this structure though there is clear evidence for its biological relevance.

The applicability of modern methods in life sciences to this structure is limited by the difficulties in gaining and preparing the required samples in a defined manner. For investigation of the mucosal pellicle's ultrastructure by TEM, biopsies represent some kind of a gold standard (Vitkov, Hannig, et al., 2002; Vitkov et al., 2001; Vitkov, Krautgartner, et al., 2002). In this context the visualization of specific structures and molecules by gold immune-labelling allows a definition of what has to be considered as mucosal pellicle in a closer sense together with simple but thorough rinses to remove the loosely associated fractions of the oral fluids (Morzel et al., 2014). Biomolecules which are not components of the mucosal cells can be labelled and identified as structural parts of the tenacious mucosal pellicle for purpose of differentiation. These ultrastructural investigations are essential for the evaluation of pellicle formation on non-solid but shedding substrates.

One prerequisite for further research on the mucosal pellicle is to establish and to validate a standardized *in situ* model for broad studies on composition and function of this layer. It is conceivable that hydrophilic materials with a high water content can be modified in order to mimic the relevant physicochemical properties of mucosal surfaces for purpose of defined oral exposure and thereby formation of an experimental *in situ* mucosal pellicle. Maybe typical mucosal components serving as binding sites could be incorporated in this experimental material which would be of relevance for other studies on surface interactions in the gastro-intestinal duct. Along this line elaborate cell culture models (over-) expressing genes for specific proteins or glycoproteins offer an interesting perspective to gain new insights into specific interactions (Ployon et al., 2016). They could be incubated *in vitro* with fresh human saliva in order to monitor the interactions. Last but not least the collection of desquamated physiological epithelial cells either gained from the oral fluids or detected within the enamel pellicle (figures 2-4) offer interesting insights. Despite these considerations it still remains difficult to gain enough pure mucosal pellicle material for elaborate analyses such as metabolomics or proteomics.

In conclusion, the combination of different methodical insights will help to solve the mystery of the mucosal pellicle.

Conclusion:

The mucosal pellicle represents an entity of high physiological relevance, but requires extensive additional research as there is only sparse knowledge on this important structure.

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Table 1: Characteristic and dominant components of the mucosal pellicle and of the enamel pellicle. The table is based on recent knowledge. For the enamel pellicle, numerous *in situ* data are available; the statement on the mucosal pellicle is based on *in vitro* and partially on *in vivo* data (Asikainen et al., 2012; Bradway et al., 1989; Bradway et al., 1992; Cardenas, Elofsson, & Lindh, 2007; Gibbins et al., 2013; Gibbins, Yakubov, et al., 2014; M. Hannig & Joiner, 2006; Kullaa et al., 2014; Morzel et al., 2014; Ployon et al., 2016; Sengupta et al., 2001)

	Enamel pellicle	Mucosal pellicle
proteins	Statherin, Histatin, Albumin, PRP, Cystatin, slgA	slgA, Cystatin; PRP, Statherin
enzymes	Amylase Lysozyme Peroxidase bacterial Glycosyltransferases	CA VI amylase
Glycoproteins and mucins	MUC1, MUC2 MUC5B	MUC5B, MUC7, MUC1, MUC4, MUC16
lipids	Palmitic-, Stearic-, Oleic- and Erucic acid;	Not yet determined

Figure 1 a: Typical characteristics of pellicle formation and initial bioadhesion on dental enamel: pellicle formation is driven by physicochemical interactions of salivary components with the tooth surface, the pellicle's ultrastructure is characterized by an electron-dense basal layer covered by granular and globular structures. The proteins and glycoproteins adsorbed from heterotypic protein aggregates are integrated in the protein network of the pellicle. This process is at least in part catalyzed by transglutaminase (TG) from the oral soft tissues which has been detected in the pellicle layer in an active conformation. There is no direct adhesion of bacteria to the tooth surface but to the specific receptors in the proteinaceous layer.

Figure 1b: Interactions during formation and maturation of the mucosal pellicle. MUC1 expressed by oral epithelial cells mediates mucin-mucin interactions with salivary mucins which may also be complexed to other proteins such as sIgA. MUC 1 is also released by minor salivary glands contributing to the formation of the oral glycocalyx (Sengupta et al., 2001). This underlines that the mucosal pellicle is a mixed coating of salivary and epithelial macromolecules providing a special interface (Kullaa et al., 2014). Other studies also identified amylase and different kinds of PRPs (Bradway et al., 1992) in the mucosal pellicle while proteins that interact mainly with inorganic components of the enamel surface, such as statherin did not appear as abundant (Gibbins et al., 2013). In the latter study, amylase showed only minimal levels of binding to epithelial cells.

Figure 2: Desquamated epithelial cells can be detected on enamel slabs exposed to the oral fluids already after 3 min (a) onto which bacteria may also be bound. They are distributed randomly in the *in situ* enamel pellicle (C. Hannig et al., 2007). Fluorescence microscopic evaluation offers the opportunity to visualize interactions of typical pellicle enzymes and bacteria with the epithelial cells. Figure 2b shows an epithelial cell (DAPI-staining) with surrounding glucans (red) and adsorbed bacterial glycosyltransferase C (green) (oral exposure time 30 min, buccal site, caries active patient). Also typical salivary enzymes such as peroxidase (green) can be detected (figure 2c, oral exposure time 8 h). Interestingly, lysozyme (green) was mainly observed around the cells but not on the bacterial cell itself (figure 2d, 2 h oral exposure). The respective methods have been published previously (Kensche, Basche, Bowen, Hannig, & Hannig, 2013). Please note that all images were acquired during different staining experiments. Different antibodies labelled with green fluorescing markers were adopted.

Figure 3: Live dead stain of free desquamated oral epithelial cells (a and b) gained from the oral fluids.

Colonies of live (green) and dead (red) bacteria are detected bound to sloughed epithelial cells gained from human saliva. The epithelial cell cytoplasm (green) and nuclei (red) are also stained.

Figure 4: TEM-images

a: TEM image of a 1-min pellicle layer (p) formed on enamel under *in situ* conditions (in the oral environment), and covered by an adherent epithelial cell. The pellicle itself is characterized by a 20 nm thick basal layer which is connected to the epithelium cell by loosely arranged fibrillar structures. On top of the epithelium cell scattered, granular- shaped pellicle-like structures (*) with an adhering bacterium (b) are detectable. An electron-dense pellicle basal layer cannot be distinguished at the epithelial cell surface. Original magnification: 49,000x; length bar = 200 nm.

b: TEM image of a 24-h bacterial biofilm - with an „integrated“ epithelial cell - formed *in situ* on enamel. Comparison of the enamel pellicle layer (p) with the surface of the epithelial cell reveals that the cell membrane is covered by a scattered pellicle-like layer (*) that resembles partly the outer globular layer of the enamel pellicle. However, on the epithelium cell surface this layer is rarely in tight connection with cell membrane, and the typical electron-dense basal layer of the enamel pellicle (p) is missing. Some adherent bacteria are marked by the letter “b”.

Original magnification: 11,000x; length bar = 2 μ m.

Please compare fig. 2, 3 and 4.

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The mucosal pellicle - an underestimated factor in oral physiology

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The mucosal pellicle is an underestimated key player in oral physiology.

The abundant and characteristic components of the mucosal pellicle include secreted soluble mucins (MUC5B, MUC7), membrane associated epithelial mucins (MUC 1), and to a lesser degree CA VI, sIgA, and cystatin.

It seems to be of completely different ultrastructure as compared with the enamel pellicle.