IN VITRO AND IN VIVO STUDIES OF
SALIVARY FILMS AT SOLID/LIQUID INTERFACES
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Malmö University, 2009
Faculty of Health and Society
I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician; he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie
ABBREVIATIONS AND SYMBOLS

\( \alpha \)  
Angle of incidence or angle of refraction

\( \Delta \)  
Ellipsometric angle related to the relative phase shift of the light components

\( \delta \)  
Thickness of unstirred layer closest to the solid/liquid interface

\( \Gamma \)  
Adsorbed amount per unit area

\( \kappa \)  
Debye screening length

\( \lambda \)  
Wavelength

\( \zeta \)  
Zeta potential

\( \nu \)  
Partial specific volume of adsorbate

\( \Psi \)  
Ellipsometric angle related to the relative amplitude change of the light components

1-DE  
One dimensional gel electrophoresis

2-DE  
Two dimensional gel electrophoresis

AFM  
Atomic force microscopy

aPRPs  
Acidic proline rich proteins

BSM  
Bovine submaxillary mucin

cac  
Critical association concentration

cmc  
Critical micelle concentration

CTAB  
Cetyl trimethylammonium bromide

D  
Diffusion coefficient

dn/dc  
refractive index increment

d\( _s \)  
Ellipsometric thickness, subscript indicates film thickness (d\( _f \)) or silicone oxide thickness (d\( _s \))

EDTA  
Ethylenediaminetetraacetic acid

FITC  
Fluorescein-5-isothiocyanate

G  
Gibbs free energy

H  
Enthalpy
HA  Hydroxyapatite
HPS  Human parotid saliva
HSMSLS  Human submandibular and sublingual saliva
HWS  Human whole saliva
IC_{50}  Concentration at which growth or activity is inhibited by 50 %
IEF  Isoelectric focusing gel electrophoresis
IPG  Immobilized pH gradient
M/A  Ratio of molar weight to molar refractivity of adsorbate
MMA  methyl methacrylate
MUC5B  Human high molecular weight salivary glycoprotein
Mw  Molecular weight
N  Complex refractive index
n_s  Refractive index (real part), subscript indicates silicon oxide
      (n_{i}), film (n_f) or bulk (n_b)
PBS  Phosphate Buffered Saline. In this work PBS= 10 mM
      phosphate buffer supplemented with 50 mM NaCl, pH 7.0
PEM  Polyelectrolyte multilayer
pI  Isoelectric point
PRP-1  Human acidic proline rich protein 1
PMMA  Poly (methyl methacrylate)
pzc  Point of zero charge
R_g  Radius of gyration
S  Entropy
SDS  Sodium dodecyl sulphate
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T  Temperature
Ti  Titanium
TIRF  Total internal reflection fluorescence
ABSTRACT

A conditioning film, the pellicle, of which many salivary proteins are important constituents, covers the surfaces present in the mouth. The pellicle forms in a selective adsorption process, and it has protective and lubricating functions as well as an influence on the adherence of oral microbes that ultimately leads to the development of dental plaque. Understanding the interactions responsible for the selective pellicle formation would make it possible to strive at creating a pellicle that serves its protective and lubricating functions and also promotes a healthy biofilm for the benefit of the individual. The aim of this research was to characterize the adsorption of salivary proteins to different types of substrates, to evaluate the influence of different protein-surface interactions on the adsorption process, and also to assess substrate dependent differences in film composition. Furthermore, as it is well known that complexes form between different salivary proteins and mucins (large glycoproteins) in bulk saliva, the aim was also to study interactions between mucins and other pellicle constituents at the solid/liquid interface. Additionally, the effects of a surfactant, sodium dodecyl sulphate (SDS), on the protein films were investigated, to evaluate the stability of the films and also the possibilities to completely remove the \textit{in vivo} formed pellicle.

The adsorption experiments were performed \textit{in vitro} using null ellipsometry, by which parameters such as adsorbed amount per unit area and average layer thickness can be obtained. Hydrophilic and hydrophobized silica were used as model substrates. The adsorption behaviour of the cationic, antimicrobial proteins lactoferrin, lactoperoxidase, lysozyme, and histatin 5 indicated that the adsorption on hydrophilic substrates was mainly driven by electrostatics, while on hydrophobized substrates hydrophobic interactions also influenced the adsorption process. Furthermore, it was shown that
sequential alternating adsorption of the anionic salivary mucin MUC5B and lactoperoxidase resulted in the build-up of multilayered structures on the surface. The rate of build-up was influenced by the surface characteristics. Sequential adsorption studies showed that neither MUC5B nor human whole saliva (HWS) was able to exchange substantial amounts of the pre-adsorbed anionic pellicle proteins acidic proline-rich protein 1 (PRP-1) or statherin, respectively. The resistance of the adsorbed mixed protein films to SDS elution depended on surface properties as well as on the number of layers adsorbed and adsorbed components. Pre-adsorbed PRP-1 was to some extent protected from SDS elution by the sequential adsorption of MUC5B to the PRP-1 film.

Pellicles formed on natural tooth enamel were collected \textit{in vivo} and investigated using two-dimensional gel electrophoresis (2-DE). Mechanically-assisted SDS elution was used to collect the \textit{in vivo} formed pellicle. The effectiveness of the collection procedure was validated \textit{in vitro} by means of mechanical removal in combination with HCl treatment. The results indicated that rubbing the tooth surfaces with fibre pellets soaked in 0.5 \% (w/v) SDS was sufficient to completely remove the pellicle from human enamel. In addition, 2-DE analysis of pellicles formed \textit{in vitro} on human enamel and the dental materials titanium and poly (methyl methacrylate) (PMMA) showed differences in composition when compared to each other, revealing that the pellicle is influenced by the substrate properties.
LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the main text by their Roman numerals. The papers are appended at the end of the thesis.

I. Adsorption behaviour and surfactant elution of cationic salivary proteins at solid/liquid interfaces, studied by in situ ellipsometry.
   **Ida E. Svendsen**, Liselott Lindh and Thomas Arnebrant

II. Lactoperoxidase and Histatin 5 – their adsorption behaviour on silica and hydrophobized silica surfaces, and implications on their role in the initial salivary film formation.
   **Ida. E. Svendsen**, Liselott Lindh and Thomas Arnebrant

III. The salivary mucin MUC5B and lactoperoxidase can be used for layer-by-layer film formation.
    Liselott Lindh, **Ida E. Svendsen**, Olof Svensson, Marité Cárdenas and Thomas Arnebrant
    *Journal of Colloid and Interface Science* 2007, 310, 74-82.

IV. Studies on the exchange of early pellicle proteins by mucin and whole saliva.
    **Ida E. Svendsen**, Liselott Lindh, Ulla Elofsson and Thomas Arnebrant
V. Validation of mechanically-assisted sodium dodecyl-sulphate elution as a technique to remove pellicle protein components from human enamel.

Ida E. Svendsen, Thomas Arnebrant and Liselott Lindh

*Biofouling* 2008, 24, 227-33.

VI. The composition of enamel salivary films is different from the ones formed on dental materials.

Ida E. Svendsen and Liselott Lindh


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**Contributions by the respondent**

In papers I, II, V and VI, I performed most of the planning and all the experimental work, including analysing the data. I was also the main contributor to writing the manuscripts. In paper III, I did most of the experimental work, and did also participate in analysing data and in discussions concerning the manuscript and writing minor parts of the paper. In paper IV, I did most of the experimental work and was also the main contributor to writing the manuscript.

**Other papers not included in the thesis:**

Human palatal saliva: adsorption behaviour and the role of low-molecular weight proteins.

Ida E. Svendsen, Thomas Arnebrant and Liselott Lindh

*Biofouling* 2004, 20, 269-77.
INTRODUCTION

Human whole saliva (HWS) is a mixture of secretions from both major and minor salivary glands, and does also contain crevicular exudate and bacterial and cellular components. The salivary secretion consists to 99% of water, and contains approximately 2 g of proteins/L fluid. Many important everyday functions occurring in the mouth, that we take for granted, can be attributed to saliva, such as lubrication to facilitate mastication, swallowing, deglutination and speaking. Furthermore, saliva maintains a steady state between tooth demineralisation and remineralisation, thereby preserving the integrity of the teeth. In addition, saliva also has an influence on the microbial growth in the mouth. All of these functions can to a large extent be assigned to the proteins of this watery secretion.

Due to the surface activity that is a general feature of proteins, salivary proteins adsorb to the different types of surfaces present intra-orally and form a conditioning film, often referred to as the pellicle. This film is believed to form by selective protein adsorption and studies have confirmed this hypothesis by the identification of particular proteins in the pellicle, whereas others were not found. The composition of the pellicle is known to influence the subsequent attachment of oral microbes, of which saliva contains approximately $10^9$/ml fluid. The microbial biofilm, frequently referred to as plaque, may in later stages progress into plaque related diseases such as caries and periodontitis. Understanding which components that are key participants in the initially formed pellicle, and also how the adsorbed components interact with each other, would make it possible to aim at enhancing the protective functions of the salivary film so that the microbial biofilm that develops would be of a healthy, protective nature only.

Studies focused on identifying pellicle constituents have reported many different types of proteins, of both anionic and cationic character as well as
proteolytically derived proteinaceous fragments\textsuperscript{7, 8}. It can be hypothesized that cationic salivary proteins may interact with anionic pellicle components and thereby increase the thickness and also the cohesiveness of the film. As numerous studies have shown that functions, such as antimicrobial activities, of several cationic salivary proteins can be retained after adsorption\textsuperscript{9-14}, the incorporation of these proteins may contribute to the defence function of the pellicle. Several studies have identified protein complex formation in bulk saliva, consisting of cationic proteins such as lactoferrin and lysozyme, combined with the large, anionic glycoproteins referred to as mucins\textsuperscript{15-17}. Also complexes between anionic salivary constituents have been identified in bulk saliva\textsuperscript{18-20}. As many of these complexes consist of pellicle proteins, such complexes may be expected to adsorb onto, and/or form on oral surfaces.
The specific objectives of this thesis have been:

To characterize the adsorption behaviour of single protein systems, specifically the cationic salivary proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5, with respect to the influence of the underlying substrate characteristics and film susceptibility to SDS elution.

To investigate systems containing several components regarding the dynamics of the adsorption process. More specifically, sequential adsorption of relevant cationic and anionic salivary proteins and possibilities for multilayer construction should be investigated. Influence of substrate characteristics as well as the film stability with respect to SDS elution should also be addressed.

To devise a method for complete removal of *in vivo* and *in vitro* formed pellicles on human enamel surfaces by mechanically assisted SDS elution. A further aim was to investigate compositional differences between *in vitro* pellicles formed on different dental materials as well as on human enamel under otherwise identical conditions.
BACKGROUND

Protein – surface interactions

The polypeptide chain of proteins is composed by, in general, 20 different amino acids linked together by peptide bonds. Depending on the side chain properties of the amino acids, they are considered as hydrophobic, polar and/or charged. The wide variety of combinations of these amino acids as well as the varying length of the peptide backbones result in very different compositions and folding of proteins, and hence properties. Proteins usually contain hydrophobic amino acids as well as polar and charged ones. This results in both an amphiphilic and an ampholytic character that will be of decisive importance when proteins in solution are adsorbed at an interface.

Protein adsorption only takes place when the Gibbs free energy (G) of the complete system decreases:

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

Generally, the characteristics of the protein, the surface and the surroundings will determine the sign and magnitude of $\Delta_{\text{ads}} G$. The interplay between protein, surface and solution depends on several different interactions that are briefly summarized below. See Norde\textsuperscript{21, 22} for more detailed descriptions.

Hydrophobic interactions

Hydrophobic interactions are considered to be one of the main driving forces for protein adsorption. The basis of this interaction is that water molecules strive to avoid non-polar components present in their vicinity. Water molecules close to a hydrophobic surface are more ordered than those
in solution and thus have lower entropy. Upon adsorption of e.g. a protein from solution, ordered water will be released from the hydrophobic substrate, as well as from hydrophobic groups of the protein, which will result in increased entropy, and hence a driving force for adsorption.

**Electrostatic interactions**

Electrostatic interactions will depend on the charges of the protein and the substrate, i.e. repulsion between similar charges and attraction between opposite charges. The structural rigidity of the proteins is also of relevance. Flexible (“soft”) proteins are generally able to structurally adapt to minimize charge repulsion with the surface, and adsorb with those amino acids that are electrostatically attracted to the surface. Hence a net negatively charged flexible protein may adsorb to a negatively charged substrate (see also effects of conformational entropy below).

**Conformational entropy**

Conformational entropy is associated with the three-dimensional structure of proteins, and is a factor that counteracts the folding of the polypeptide chain. Studies have indicated structural rearrangements in proteins upon adsorption, which both may increase (i.e. more unordered protein structure) or decrease (e.g. increased secondary structure at apolar surfaces) the conformational entropy. Generally, changes in conformational entropy may be important for adsorption of larger, structurally less stable proteins.

**van der Waals interactions**

van der Waals interactions are based on interactions between permanent and/or induced dipoles. These types of interactions usually favour protein adsorption, however, they are short ranged and rather weak when compared with hydrophobic and electrostatic interactions.

**Hydrogen bonds**

Hydrogen bonds occur between an electronegative atom and hydrogen atoms usually bound to oxygen or nitrogen. The strength of these interactions with respect to protein adsorption is usually regarded to be in the same range as van der Waals interactions.

Also the composition of the ambient solution is of importance for protein adsorption. The concentration of ions as well as their valency, which both
affect the Debye screening length ($\kappa^{-1}$), may to a large extent determine the adsorption process, as it will affect the range of the electrostatic interactions between the protein and the surface, as well as other intra- and intermolecular interactions. The solution pH is also of relevance as it controls the ionisation of many amino acid residues.

Natural and artificial surfaces in the oral cavity

Different types of surfaces of both hard and soft character, and composed of both natural and artificial materials, may be present in the oral cavity. Large differences in chemical composition, charge, microstructure and surface free energy are observed for these surfaces. The following section focuses on human enamel, as well as on the dental materials titanium and poly (methyl methacrylate) [PMMA] that were used in studies included in this thesis.

Human enamel

Dental enamel is the outer layer of the tooth crown, constituting the hardest tissue present in the human body. It is highly mineralised, and the main mineral component of enamel (approximately 95 %) is hydroxyapatite (HA), $\text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2$. The calcium ions have a stronger tendency to dissolve in aqueous solutions than the phosphate ions, which results in excess of the phosphate ions ($\text{HPO}_4^{2-}$ and $\text{H}_2\text{PO}_4^-$) at the interface, giving the enamel surface a net negative charge. Counter-ions will then “coat” this surface, which results in an electric double layer (Figure 1).

An organic matrix is present between the HA prisms of the enamel. This matrix (constituting $\sim 1 \%$ of the enamel), consists of specific proteins, mostly amelogenins, but also e.g. ameloblastin, enamelin and enamel proteases. In addition, the dental enamel also carries small amounts of water ($\sim 4 \%$).

Reported values of surface free energy of pure enamel are $77 \pm 10$ to $87 \pm 6$ mJ/$\text{m}^2$, indicating complete water wetting, which also has been reported for HA. The point of zero charge (pzc) of enamel has been reported to be 4.4-5.0. HA particles have been reported to have pzc values between 6.5-7.3.
Titanium

Titanium (Ti) is a frequently used biomaterial, not only for dental reconstructions and implants but also as implants in orthopaedic applications, such as hip joint replacements. When titanium is exposed to air or aqueous solutions, an oxide layer is spontaneously formed on the surface, and TiO$_2$ is the most common oxide of titanium. A typical Ti dental implant is formed to resemble a single tooth root and has a roughened or smooth surface. Modification of the surface properties of titanium, such as surface roughness and surface free energy, is known to influence osseointegration. Titanium has many properties that make it an excellent biomaterial; it is e.g. nontoxic and mechanically resistant. It has a pI at approximately 6 and a high surface free energy (water contact angle <8°). Titanium covered with TiO$_2$ has been investigated in detail regarding biocompatibility with respect to interactions with blood, but few studies have been carried out on saliva interactions.

PMMA

Poly(methyl methacrylate) (PMMA; (C$_5$O$_2$H$_8$)$_n$, Figure 2) is a polymer used in e.g. dentures and bone cements. It is highly biocompatible and low-priced. PMMA has a low surface free energy (water contact angle: 75°).
and, considering its molecular structure, it is uncharged provided that the ester functionalities are intact.

Although PMMA is biocompatible, the toxic monomer of PMMA, methyl methacrylate (MMA), may remain after the initial polymerization. However, after completion of the polymerization process, only small amounts of monomer are released during the lifetime of the prosthesis, and the toxic effects are therefore almost negligible.

Figure 2. Drawing of the molecular structure of a PMMA subunit. $n$=number of subunits.

The salivary film formation

Numerous in vitro studies have been performed to elucidate the physico-chemical phenomena in the pellicle formation (see e.g.\textsuperscript{38-41}); e.g. protein adsorption from HWS and salivary fractions, as well as studies of adsorption of single salivary proteins to model surfaces. It is generally accepted that after approximately 1-2 hours of film formation, plateau values regarding adsorbed amount and thickness of the salivary film has been reached, depending somewhat on experimental conditions\textsuperscript{4, 38}. A relatively wide variation in adsorption behaviour exists among different salivary fractions and purified salivary proteins\textsuperscript{38}. However, a general feature is that larger amounts are usually adsorbed on hydrophobized surfaces compared to hydrophilic surfaces\textsuperscript{38, 40-42}. AFM studies of adsorbed films from HWS have shown that both hydrophobized and hydrophilic silica are completely covered by a salivary film, however, the size and shape of adsorbed components, as well as the density of the film, are substrate dependent\textsuperscript{40}.

From investigations on the adsorption of individual salivary protein, fundamental data concerning driving forces for adsorption as well as substrate affinity and specific protein-substrate interactions may be elucidated. Such information is essential for the analysis of data obtained from adsorption
studies of solutions containing two or more components. Since statherin and acidic proline-rich proteins (aPRPs) are known components of the initial pellicle, the adsorption behaviour of statherin, aPRPs, and also salivary mucins have attracted quite a bit of attention (see e.g. 40, 41, 43-48). The salivary mucin MG1, which consists primarily of MUC5B49, is also known as a pellicle constituent, and was recently shown to have potential as a biomaterial coating, suppressing neutrophil adhesion and activation50. Furthermore, by means of adsorption experiments and streaming potential studies, it has been shown that the in vitro formed pellicle contains a significant fraction of positively charged proteins51, 52, which merits the investigation of the adsorption of individual cationic proteins present in saliva. The cationic proteins lysozyme and lactoferrin have been studied extensively as model proteins for adsorption on different types of surfaces53-57, and adsorption studies of lactoperoxidase and histatin 5 have also been performed58-60.

Most studies have concluded that the pellicle to a large extent is irreversibly adsorbed. Adsorption experiments performed in vitro often include a final step of buffer rinsing or surfactant addition, to get an estimate of the binding properties of adsorbed components. In the case of adsorption from complex solutions like saliva, the differences in elutable fractions on different surfaces could indicate compositional differences or multiple states of binding.

The pellicle composition and function

Collection of the pellicle has been the objective in numerous studies6, 61-65, and complete pellicle elution has been shown to be a difficult task. However, many different types of proteins, as well as carbohydrates and lipids, have been identified as pellicle constituents6. Proteins such as lysozyme, aPRPs, amylase, albumin, histatins, statherin, lactoferrin, mucins, cystatins and immunoglobulins are among the most frequently identified proteins in the pellicle on enamel6, 66, and some of these have also been found in pellicles on different dental materials66-70. The physiological roles of these proteins include maintaining calcium homeostasis, antimicrobial activity and lubrication. In addition, the pellicle has been shown to act as a diffusion barrier to acids71, a function not attributed to specific components but rather to the whole film.

Microbial adherence to oral surfaces, leading to the formation of plaque or a microbial biofilm, is in its initial stage influenced by the pellicle composition, due to specific (receptor-donor) and non-specific interactions72-74. Factors such as dental hygiene and frequency and type of food intake will influence the
pellicle composition, as well as the survival and proliferation of the biofilm community.

**Dynamics of salivary films**

**Competitive adsorption and sequential exchange**

The pellicle formation is initiated by the adsorption of specific small proteins, known as pellicle precursors, e.g. statherin, histatins and aPRPs. A continuous adsorption then takes place, which presumably involves interactions between components in saliva and the precursor proteins, and to patches on the surface that are still uncovered.

Studies on sequential salivary adsorption processes are rather scarce. However, as both saliva and blood are examples of complex fluids containing a wide range of e.g. proteins and ions, it may be anticipated that a process analogous to “the Vroman effect” (adsorption and exchange of components in blood dependent on e.g. concentration and molecular weight) takes place on surfaces in the oral cavity. It has been shown that the high molecular weight salivary mucin MUC5B adsorbs rather slowly, indicating that it does not play an important role during the initial pellicle formation, but it nevertheless appears in the film at later stages. MUC5B has a higher adsorption affinity for HA compared to smaller pellicle precursor proteins such as aPRPs and statherin, indicating its potential to exchange these components at the interface. Furthermore, a recent study indicated that α-amylase has the potential to replace lysozyme on fluoroapatite particles, and albumin on silica surfaces.

**Multilayer formation**

Outside the oral field, the interest in polyelectrolyte multilayer (PEM) films is large, as such structures have the potential to be used as e.g. biomaterial coatings, thus creating more biocompatible surfaces. In general, oppositely charged polyelectrolytes are used for the layer-by-layer deposition process originally developed by Decher et al. The driving force for the build-up of these films is the charge reversal that occurs after each adsorption step (alternating between a positive and a negative charge) resulting in an electrostatic attraction between each layer. A film with arbitrary thickness can be constructed simply by varying the number of adsorption cycles.
Factors such as pH and ionic strength will also influence the build-up behaviour\(^79\).

Several publications using proteins and polypeptides in PEMs have been reported, and with respect to oral significance, lysozyme and mucins, respectively, have been used to build multilayered films successfully with oppositely charged polyelectrolytes\(^81\)\(^\text{-}83\), and enzymatically active lactoperoxidase has been incorporated into PEM films in so-called enzyme nanoreactors\(^84\). Also, antimicrobial peptides have been inserted in PEM films to protect surfaces from bacterial colonization\(^85\), a function relevant for the oral cavity. Embedding components with specific functions in multilayered films could increase the depot of these active constituents and hence promote specific functions of the film.

**Action of surfactants**

The interactions between an adsorbed protein film and a surfactant can be used as a measure of the strength of the protein–surface interaction\(^86\). The degree of removal depends on the surfactant type as well as on its concentration. Also, the protein and substrate properties are of importance, as is the adsorption time\(^87\). Generally, sodium dodecyl sulphate (SDS), which is a negatively charged surfactant, is more effective in the removal of protein and salivary films, compared to cationic surfactants (e.g. CTAB) and other elution agents (e.g. EDTA)\(^61\)\(^\text{-}63\),\(^86\),\(^88\).

Surfactants are introduced e.g. in tooth pastes, thereby combining the cleaning effect of the surfactant with the mechanical cleansing action of the toothbrush. Although the mechanical action has been indicated to be of main importance for salivary film removal\(^62\), the type of surfactant employed is also of significance\(^61\)\(^\text{-}63\). In general, at concentrations close to the critical micelle concentration, cmc, of the surfactant (in reality the critical association concentration, cac, of the surfactant-protein system), the elutability reaches its maximum. An example is SDS elution of HWS films on HA in vitro\(^61\), where a SDS concentration above cmc had no further effect on the desorption\(^89\). It is often observed that the surfactant removes parts of the adsorbed protein films, indicating a complex film composition and/or multiple states of binding\(^90\). Variations in the desorbable fraction of proteins and saliva have been observed on hydrophilic and hydrophobized substrates\(^40\),\(^42\),\(^86\),\(^88\). For saliva, binding to hydrophobic surfaces typically is stronger and may involve exposure of hydrophobic domains of the protein to the surface. Such
conformational changes may result in a decreased removable fraction\textsuperscript{22}, however, also the film composition is of relevance\textsuperscript{91}.

SDS has been used extensively as a component in e.g. toothpastes, usually in concentrations up to 2\%\textsuperscript{92}. Studies have shown that SDS may be harmful to mucosal surfaces\textsuperscript{92, 93}, and that SDS treatment reduces the lubrication properties of salivary films\textsuperscript{84, 95}. On today’s market, many different types of surfactants are used in oral care products, such as sodium lauryl sarcosinate and polyethylene glycol esters that generally are less irritating compared to SDS. In the studies presented here, SDS was used as a model surfactant to investigate the stability of the adsorbed films.

Two models have been suggested to be the main modes of protein elution by surfactants\textsuperscript{96}:

\begin{center}
\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Schematic illustration of the formation of soluble complexes (a) and the replacement mechanism (b). Adapted from\textsuperscript{96}. Not drawn according to scale. \(\ddagger\) = protein, \(\bullet\) = surfactant micelle, \(\ast\) = surfactant monomer.}
\end{figure}
\end{center}

\textit{Formation of soluble complexes (Figure 3a)}

The surfactant interacts with the adsorbed proteins, generating complexes that may be electrostatically repelled from the surface (in case of similarly charged surfactant and substrate). This is typical for SDS, which is known to interact strongly with proteins\textsuperscript{86}. Conformational changes and/or higher solubility of the formed complexes may also explain the removal. The surfactant itself does not adsorb to the surface, cf. SDS on a negatively charged surface.
Protein replacement (Figure 3b)

The surfactant has a higher affinity for the surface compared to the adsorbed proteins, which results in an exchange of components on the surface. As water soluble surfactants, such as SDS, are expected to be reversibly adsorbed on e.g. hydrophobic surfaces, a subsequent rinse removes the surfactant from the surface. The surfactant does not interact with the adsorbed proteins.

In many systems, a combination of the two models described above may be operational, e.g. when the surfactant interacts with both the substrate and the adsorbed proteins.

Pellicle proteins of interest

Some specific salivary proteins have been selected for adsorption studies in the present thesis. The selection was based primarily on the following criteria: (i) their presence in the pellicle (ii) important functions of the proteins (iii) suitability as model proteins in adsorption studies. Short descriptions of the proteins chosen are given in the materials and methods section below.
MATERIALS AND METHODS

Surfaces

Enamel

Human teeth, extracted due to orthodontic treatment, were used as enamel samples. Previous to use, the teeth were pumiced using a rubber cup and fine grade pumice, followed by extensive rinsing in water and drying in a stream of filtered air. The surfaces were used directly after cleaning.

Titanium

Titanium surfaces were obtained from Nobel Biocare AB (Göteborg, Sweden). The surfaces were cleaned by extensive rinsing in water, ethanol and water, and dried with filtered air, after which the surfaces were plasma cleaned in low pressure air for 5 minutes in a glow-discharge Harrick Plasma cleaner (Model PDC-32G, Harrick Scientific Corporation, Ossining, NY, USA). The surfaces were used immediately after plasma cleaning.

PMMA

PMMA (Microdent, Type 1/class 1 according to ISO 1567:1988 (E)) was obtained from Forshaga Dental depå AB (Forshaga, Sweden). The surfaces were cast and treated according to the instructions from the manufacturer. Before use, the surfaces were cleaned by rinsing in water, followed by rinsing in ethanol and finally water, after which they were dried with filtered air.
Silica surfaces

Silicon slides, used for the ellipsometry and AFM experiments, were obtained from Okmetic OY (Espoo, Finland). The surfaces, which had an oxide thickness of approximately 300 Å, were plasma cleaned for 5 min as described for the titanium surfaces. Subsequently, the surfaces were made hydrophilic by 5 minutes of gentle boiling in a solution of NH$_4$OH (25%):H$_2$O (30%):H$_2$O (1:1:5 v/v), followed by water rinsing and another 5 minutes of gentle boiling in a solution of HCl (37%):H$_2$O (30%):H$_2$O (1:1:5 v/v). Finally the surfaces were rinsed in water and ethanol and stored in ethanol. Just prior to use, the surfaces were rinsed in water, ethanol and water, dried in a stream of nitrogen gas followed by 5 minutes of plasma cleaning. Hydrophobized silica surfaces were prepared from the hydrophilic substrates by means of liquid phase silanization. The surfaces were rinsed in trichloroethylene before immersion in a 0.05% (v/v) dichlorodimethylsilane solution in trichloroethylene for 1 h. Subsequently the surfaces were rinsed in trichloroethylene followed by ethanol, and stored in ethanol. Before experiments, the hydrophobized surfaces were rinsed in water, ethanol and water and then dried with nitrogen gas. Both types of surfaces have a reported ζ potential of -45 ± 5 mV in 1 mM NaCl pH 7.0 and water contact angles of <10° for hydrophilic silica and 103° for hydrophobized substrates (advancing contact angles).

Techniques aimed at investigating interactions taking place at solid/liquid interfaces usually require surfaces that have well-defined features, such as low surface roughness and specific optical or mechanical properties, to be able to record e.g. protein adsorption and thus accurately interpret adsorption mechanisms (see e.g. 100). Dental enamel is highly heterogeneous in terms of surface composition and surface structure. These features make enamel difficult to apply to the techniques used here (ellipsometry, AFM and TIRF, see below) without treatments that might change the surface characteristics. The wide range in wettability spanned by the model silica surfaces include surface wettabilities of many types of surfaces that may be found in the oral cavity, which justifies the choice of model substrates.

Glass surfaces

Glass slides (Gold Seal Rite-on, Microslides, Clay Adams, NY, USA) were used for the TIRF experiments (see below). They were cleaned and modified as described for the silica surfaces to obtain hydrophilic and hydrophobic
characteristics, respectively. Reported water contact angles are <10° for hydrophilic glass and 103° for hydrophobized glass (advancing contact angles).

**Saliva and salivary proteins**

**HWS**

Unstimulated HWS was collected by drooling into ice-chilled tubes for approximately 10 min, as described by Dawes. The donors were considered to be in good general and oral health. The collection was performed in the morning, at least 2 hours after food intake and tooth brushing. The collection was a standardized procedure and has been shown to yield reproducible results. All collections of HWS and pellicle (see below) were approved by the Committee for research ethics at Lund University (approval No LU518-02).

For the adsorption experiments (paper IV), saliva was diluted to 10%. It has previously been shown that the major part of the adsorbed film had been formed at this concentration, and experiments performed at this concentration also avoided problems due to light scattering. No significant differences in total adsorbed amounts and film thicknesses were observed between different donors at identical saliva concentrations, however, qualitative differences have been indicated.

**Proteins**

Background information of the investigated proteins is given below, with some characteristics summarized in Table 1. The commercially available proteins were used as received.

The adsorption behaviour of the cationic proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5 was investigated using concentration limits reported for saliva (Table 1). Although not all of the proteins used were of human origin, their characteristics were similar, and therefore their adsorption behaviour were assumed to resemble that of the respective components in saliva.

**Lactoferrin**

Lactoferrin is a non-enzymatic glycoprotein, with iron-chelating properties, thereby depriving microbes of this essential nutrient. It also has shown e.g.
anti-inflammatory, anti-cancer and antiviral features\textsuperscript{106}, as well as being present in the pellicle\textsuperscript{107, 108}. Human milk lactoferrin (L0520) was obtained from Sigma-Aldrich Sweden AB (Stockholm Sweden).

\textit{Peroxidase}

Salivary peroxidase is a glycoprotein which catalyses the oxidation of specific molecules (generally thiocyanate ions), resulting in the formation of highly reactive derivatives, such as hypothiocyanite/hypothiocyanous acid, which are toxic to several types of microorganisms\textsuperscript{11, 109-112}. Peroxidase has been identified in the pellicle\textsuperscript{12, 63}, and retains its activity after adsorption\textsuperscript{12}. Bovine milk lactoperoxidase (L8257) was obtained from Sigma-Aldrich Sweden AB.

\textit{Lysozyme}

Lysozyme exerts its antimicrobial functions by e.g. inducing bacterial lysis leading to cell death\textsuperscript{110, 111}. Lysozyme has been identified in pellicles formed on both natural and artificial dental surfaces\textsuperscript{31, 43, 46, 108, 113}, and studies have further shown that lysozyme remains active when incorporated in the pellicle\textsuperscript{\textsuperscript{13}}. Lysozyme from chicken egg white (Sigma-Aldrich Sweden AB (L-6876)) was used in the adsorption experiments.

\textit{Histatin 5}

Histatins are a family of histidine-rich proteins that are known to have antifungal activities\textsuperscript{114}. Histatin 5 is mostly known to inhibit the growth of \textit{Candida albicans}, the main fungi responsible for candidiasis\textsuperscript{114}. Histatin 5 has shown to adsorb to different materials\textsuperscript{9, 58, 60}. Synthetic histatin 5 was purchased from American Peptide Company Inc. (Sunnyvale, CA, USA).

\textit{MUC5B}

MUC5B is one of the major mucins present in saliva, and known primarily for its lubricating functions\textsuperscript{115}. It has been identified in the 2 h old pellicle\textsuperscript{6, 116}. It is a large oligomeric molecule, where the monomers are joined by disulphide bridges. MUC5B has been reported to be composed of approximately 80% carbohydrates, of which approximately 55% are neutral and 45% are acidic (sulphated or sialylated)\textsuperscript{117} resulting in an expected low pI of MUC5B.

MUC5B was purified from HWS, as previously described\textsuperscript{43, 118}. Studies on the initial adsorption rate indicated that the MUC5B preparation consisted of both intact mucin macromolecules as well as subunits and T-domains\textsuperscript{45}. 
Statherin

Statherin is a small protein, known mainly for its involvement in maintaining calcium homeostasis\textsuperscript{119}. In addition, statherin has been shown to have lubricating abilities\textsuperscript{45, 120} and is also recognized to mediate microbial colonization on HA surfaces\textsuperscript{121}. It has been reported in several studies to be present in the pellicle\textsuperscript{63, 108, 122}.

Proline-rich protein 1 (PRP-1)

PRP-1 belongs to the family of acidic proline-rich proteins and is known to be involved in maintaining the calcium homeostasis of saliva, e.g. inhibiting crystal growth\textsuperscript{119}, and also in lubrication\textsuperscript{45}. PRP-1 is known to adsorb in the initial phase of pellicle formation\textsuperscript{4} and has been identified in the pellicle\textsuperscript{61}.

Statherin and PRP-1 was purified from human parotid saliva (HPS) as described in Li \textit{et al.}\textsuperscript{123} and Lindh \textit{et al.}\textsuperscript{44}. The identities and purities of both proteins were confirmed by gel electrophoresis, NH\textsubscript{2}-terminal amino acid sequencing and bacterial binding properties\textsuperscript{123}.

Estimations of diffusion coefficients

As no diffusion coefficients of histatin 5, statherin and PRP-1 could be obtained in the literature they were estimated from a graph of known diffusion coefficients vs. molecular weights of a series of well defined proteins, as previously described\textsuperscript{44, 124}. As the proteins comprised in this graph are of globular structure, the diffusion coefficient of histatin 5, statherin and PRP-1 may be overestimated.
Table 1. Some characteristics of the investigated proteins

<table>
<thead>
<tr>
<th></th>
<th>Lactoferrin</th>
<th>Lactoperoxidase</th>
<th>Lysozyme</th>
<th>Histatin 5</th>
<th>MUC5B</th>
<th>Statherin</th>
<th>PRP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. in saliva ( ^\text{a} ) ( (\mu M) )</td>
<td>( \leq 0.3 )</td>
<td>( \leq 0.2 )</td>
<td>( \leq 14.0 )</td>
<td>( \leq 15.0 ) (HSMSLS)</td>
<td>( \leq 0.1 )</td>
<td>( \leq 29.6 ) (HPS)</td>
<td>( \leq 11.3 ) (total)</td>
</tr>
<tr>
<td>Conc. in saliva ( ^\text{a} ) ( (\mu g ml^{-1}) )</td>
<td>( \leq 24 )</td>
<td>( \leq 13 )</td>
<td>( \leq 200 )</td>
<td>( \leq 45 ) (HSMSLS)</td>
<td>( \leq 865 )</td>
<td>( \leq 160 ) (HPS)</td>
<td>( \leq 180 ) (aPRPs)</td>
</tr>
<tr>
<td>Mw ( kDa )</td>
<td>78</td>
<td>78 ( ^\text{f} )</td>
<td>14.5 ( ^\text{f} )</td>
<td>3.0</td>
<td>13500 ( ^\text{g} ) 2100 ( ^\text{g} )</td>
<td>5.4</td>
<td>16.0</td>
</tr>
<tr>
<td>pI</td>
<td>( ^\text{e} \sim 9 )</td>
<td>8-10</td>
<td>( ^\text{e} \sim 11 )</td>
<td>10.3</td>
<td>6.2 ( ^\text{e} )</td>
<td>4.2</td>
<td>4.7</td>
</tr>
<tr>
<td>Structure</td>
<td>Globular, glycosylated</td>
<td>Globular, glycosylated</td>
<td>Globular</td>
<td>Flexible</td>
<td>Flexible, glycosylated</td>
<td>Flexible, phosphorylated</td>
<td>Flexible, phosphorylated</td>
</tr>
<tr>
<td>Dimensions (Å)</td>
<td>47<em>47</em>190</td>
<td>55<em>81</em>78</td>
<td>30<em>30</em>45</td>
<td>R( _g ) = 7</td>
<td>R( _g ) =1780 ( ^\text{g} ) R( _g ) =63 ( ^\text{g} )</td>
<td>R( _g ) =9</td>
<td>R( _g ) =18</td>
</tr>
<tr>
<td>Monolayer ( ^\text{g} ) ( (nmol m^{-2}) )</td>
<td>19-75 ( ^\text{h} )</td>
<td>27-39 ( ^\text{h} )</td>
<td>123-184 ( ^\text{h} )</td>
<td>1000</td>
<td>0.015 ( ^\text{h} ), 0.1 ( ^\text{h} )</td>
<td>587</td>
<td>147</td>
</tr>
<tr>
<td>Monolayer ( ^\text{g} ) ( (mg m^{-2}) )</td>
<td>1.5-5.9 ( ^\text{h} )</td>
<td>2.1-3 ( ^\text{h} )</td>
<td>1.8-2.7 ( ^\text{h} )</td>
<td>3.0</td>
<td>0.2 ( ^\text{h} ), 0.3 ( ^\text{h} )</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Diffusion coefficient ( (10^{-11} m^2 s^{-1}) )</td>
<td>6.0</td>
<td>5.9</td>
<td>10.4</td>
<td>16.0</td>
<td>0.19 ( ^\text{h} )</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Net charge density ( ^\text{g} ) ( (mmol/g) ) ( ^{^\text{i,j}} )</td>
<td>( +0.15 )</td>
<td>( +0.051 )</td>
<td>( +0.56 )</td>
<td>( +1.7 )</td>
<td>( -0.083 ), ( -0.058 )</td>
<td>( -0.56 )</td>
<td>( -0.63 )</td>
</tr>
<tr>
<td>Net charge density ( ^\text{g} ) ( (mol/mol) ) ( ^{^\text{i,j}} )</td>
<td>( +12 )</td>
<td>( +4 )</td>
<td>( +8 )</td>
<td>( +5 )</td>
<td>( -49 ), ( -155 )</td>
<td>( -3 )</td>
<td>( -10 )</td>
</tr>
<tr>
<td>Aliphatic index ( ^\text{g} ) ( ^{^\text{i,j}} )</td>
<td>75</td>
<td>81</td>
<td>65</td>
<td>4</td>
<td>-</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>References</td>
<td>74, 109, 115</td>
<td>110, 119, 126</td>
<td>120, 127, 128</td>
<td>129-131</td>
<td>132-134</td>
<td>91, 77, 119</td>
<td>77, 119</td>
</tr>
</tbody>
</table>

\(^{^\text{a}}\) Concentrations are in HWS unless otherwise stated.
\(^{^\text{b}}\) The net charge at pH 7.0 of the protein backbone divided by the molecular mass of the protein backbone. For statherin and PRP-1, the phosphos- 
esines were also taken into account.
\(^{^\text{c}}\) The relative volume occupied by aliphatic side chains.
\(^{^\text{d}}\) The molecular weight of the component used in the present studies (obtained from literature or Swiss-Pro 
database provided by The Swiss Institute of Bioinformatics), although closely resembling the true molecular weight of the salivary counterparts, 'suck-on-end-on', RCSB Protein Data Bank 2GJ1
\(^{^\text{e}}\) Whole mucin and subunit.
\(^{^\text{f}}\) Theoretical value of the amino acid sequence only, glycosylated domain.
Amounts corresponding to monolayer coverage

For all investigated proteins, the adsorbed amounts corresponding to monolayer coverage, was calculated from the formula:

$$\Gamma = \frac{1}{AN_a} \quad \text{Eq. 1}$$

where $\Gamma$ is the adsorbed amount per unit area (mol·m$^{-2}$), A is the area per molecule (m$^2$) and $N_a$ is Avogadro’s constant ($6.022 \times 10^{23}$·mol$^{-1}$). Multiplying Eq. 1 with the molecular weight of the protein changes the unit of $\Gamma$ to mass per unit area. Dimensions of the globular proteins lactoferrin, lactoperoxidase and lysozyme are known from the literature (Table 1).

The dimensions of histatin 5, statherin and PRP-1 are to the author’s knowledge unknown. As these proteins can be considered as flexible, fairly unstructured molecules, amounts corresponding to monolayers of these proteins were estimated by assuming hexagonally close packed spheres with a cross sectional area of $\pi R_g^2$, the same assumption was applied to monolayer calculations for MUC5B based on its reported radius of gyration ($R_g$). $R_g$ was calculated for histatin 5, statherin and PRP-1 (assuming theta solvent) using the following formula:

$$R_g^2 = \frac{nl^2}{6} \quad \text{Eq. 2}$$

where n is the number of amino acids and l is the length of each amino acid backbone (approximately 3.5 Å). The $R_g$ was used to obtain a first approximation of the dimensions. The hydrodynamic radius, i.e. the radius of the hydrated molecule, would be a more accurate estimation of the molecular dimensions and expected to be slightly larger than $R_g$.

General

All water used was processed in an Elgastat UHQ II unit (Elga Ltd, High Wycombe, Bucks, England) (Papers I-III, and V-VI), or in a MilliQ unit (Millipore, Bedford, MA, USA, including an ion exchange active carbon adsorption and reverse osmosis) (Paper IV). All ethanol used was of at least...
96% purity. All chemicals were of at least analytical grade (VWR International, Stockholm, Sweden; Primalco, Helsinki, Finland; Amersham Biosciences, Uppsala, Sweden; Bio-Rad, Sundbyberg, Sweden; Sigma-Aldrich Sweden AB, Stockholm, Sweden). The buffer solution used for experiments described in papers I-IV, PBS, was a 10 mM phosphate buffer supplemented with 50 mM NaCl, with a pH of 7.0. The buffer composition had a calculated ionic strength of approximately 80 mM. SDS was purchased from Sigma-Aldrich Sweden AB (L6026), and was used as received. It was dissolved in PBS to a concentration of 17 mM for experiments performed with ellipsometry, AFM and TIRF. The cmc of SDS in PBS has been estimated to be 1.95 mM. For pellicle collections, SDS was diluted in UHQ. The cmc of SDS in water is known to be 8.3 mM (i.e. 0.25% (w/v)).

In situ null ellipsometry

Null ellipsometry is an optical technique based on analysing changes in the polarisation of light upon its reflection at an interface. Ellipsometry can be used to gain detailed information on adsorbed films with respect to e.g. adsorbed mass per unit area and average film thickness with a resolution down to approximately 0.05 mg×m⁻² and a few Å, respectively.

Figure 4. Ellipsometer set-up.

This non-destructive technique is time-resolved, allowing information on the kinetics of the adsorption to be registered without interfering with the adsorption process. The measurements are done in a temperature controlled liquid cell, with continuous stirring, and it is also possible to exchange the
solution in the cell by continuous rinsing, so that e.g. reversibility of adsorption may be monitored.

The instrument set-up is shown in Figure 4. Light from a light source (in our case a xenon arc-lamp, filtered to $\lambda \approx 4429$ Å, for experiments described in paper IV: $\lambda \approx 4015$ Å) first passes a polariser which results in linearly polarised light. The plane polarised light then passes the compensator (a quarter-wave plate), which induces a relative phase shift in the light vectors (light oscillating parallel and perpendicular to the fast and slow axis), rendering it elliptically polarised. The reflection at the solid/liquid interface further changes the polarisation, resulting in an additional relative phase shift and amplitude change. The polariser can be adjusted in such a way that the ellipticity prior to reflection results in linearly polarised light after reflection, and can be “extinguished” by the second polariser, called the analyser. The light intensity after the analyser is recorded by the detector. During a measurement, the optical components (usually the polariser and analyser, while the compensator azimuth is kept constant at $\pm 45^\circ$) are adjusted by stepping motors to minimize the light reaching the detector. There are four sets of positions for the polariser, analyser and compensator that all result in light minima. These four settings are known as the four zones, and by averaging the azimuths obtained in all zones, most optical imperfections, such as misalignment of the sample and tilted optical components, can be corrected for.$^{137}$ Averaging over two zones, keeping the compensator azimuth fixed, corrects for the major optical imperfections. From the azimuths of the polariser and analyser obtained at the nulling settings, the ellipsometric angles $\Psi$ and $\Delta$ can be calculated. The relative amplitude ratio ($\Psi$) is obtained from the azimuth of the analyser, while the relative phase shift ($\Delta$) is obtained from the azimuth of the polariser. Four different sets of $\Psi$ and $\Delta$ can be obtained according to Table 2:

Table 2. Conversion from azimuth of the analyser ($A$) and polariser ($P$) into the ellipsometric angles $\Psi$ and $\Delta$ for the different zones.$^{137}$

<table>
<thead>
<tr>
<th>Zone</th>
<th>Azimuth of the compensator</th>
<th>$\Psi$</th>
<th>$\Delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$-45^\circ$</td>
<td>$A$</td>
<td>$2P+90^\circ$</td>
</tr>
<tr>
<td>2</td>
<td>$+45^\circ$</td>
<td>$A$</td>
<td>$-90^\circ$-$2P$</td>
</tr>
<tr>
<td>3</td>
<td>$-45^\circ$</td>
<td>$180^\circ$-$A$</td>
<td>$2P$-$90^\circ$</td>
</tr>
<tr>
<td>4</td>
<td>$+45^\circ$</td>
<td>$180^\circ$-$A$</td>
<td>$90^\circ$-$2P$</td>
</tr>
</tbody>
</table>
The instrument used was a Rudolph thin film ellipsometer type 43603-200E (Rudolph Research, Fairfield, NJ, USA), automated according to Cuypers\textsuperscript{138}. For each experiment, a two-zone (or a four-zone, paper IV) surface calibration, was carried out in two ambient media (air and PBS). A three-layer optical model (ambient, surface oxide layer and bulk surface) was used to determine all unknowns in the clean system, i.e. the complex refractive index ($N_0 = n_0 - ik_0$) of the silicon and the real part of the refractive index ($n_1$) and thickness ($d_1$) of the silicon oxide layer (the oxide layer was assumed to be transparent, i.e. $k_1=0$). For the calculations, the dimethylsilane layer on the hydrophobized silica was assumed to be included in the oxide layer. This simplification will result only in a minor error due to the minor thickness of the dimethylsilyl layer (a few Å)\textsuperscript{139}.

After surface characterization, the sample was added to the cell, and $\Psi$ and $\Delta$ were recorded \textit{in situ} in real time. When the optical properties of the substrate and the ambient media is known, the average layer thickness ($d_f$) and refractive index ($n_f$) of the protein film can be solved by numerical iteration from the changes in $\Psi$ and $\Delta$\textsuperscript{140}, assuming a homogeneous film. The evaluation of the adsorbed protein film was performed by using a four-layer optical model, with isotropic media and planar interfaces (Figure 5).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure5.png}
\caption{Schematic illustration of the 4-layer model. $\alpha$ = angle of incidence or angle of refraction.}
\end{figure}

If the film is inhomogeneous, or if the level of adsorption is low, resulting in only minor changes in $\Psi$ and $\Delta$, the refractive index ($n_f$) and average thickness ($d_f$) estimations of the film are unreliable\textsuperscript{141}. The relative errors in $n_f$ and $d_f$ are relatively high for low adsorbed amounts ($\Gamma < 0.5$ mg m\textsuperscript{-2}), however, for $\Gamma > 1$...
mg m$^2$, the error is only 5-10%\textsuperscript{142}. Errors in n, and d, co-vary, resulting in a much higher accuracy for the calculated adsorbed mass, even at small changes in $\Psi$ and $\Delta$ (an error of approximately 15\% at $\Gamma = 0.1$ mg m$^2$ and less than 1\% for $\Gamma = 2$ mg m$^2$)\textsuperscript{142}.

From the film thickness and the refractive index, the adsorbed amount can be calculated according to the model by Cuypers et al.\textsuperscript{141} (Papers I-II and IV):

$$\Gamma = \frac{0.3 df(n_f)}{(A/M) - \nu \left( (n_b^2 - 1)/(n_c^2 + 2) \right)} (n_f - n_b) \quad \text{Eq. 3}$$

where

$$f(n_f) = \frac{n_f + n_b}{(n_f^2 + 2)(n_b^2 + 2)} \quad \text{Eq. 4}$$

Cuypers' model is based on the difference in refractivity of different functional groups. This is described by the ratio of the molar weight to the molar refractivity (M/A) of the adsorbate and its partial specific volume ($\nu$). Values of M/A and $\nu$ used (papers I-II and IV) were 4.10 g ml$^{-1}$ and 0.75 ml g$^{-1}$, respectively. These values are averages for proteins, and have been used previously in studies of adsorption from saliva and salivary components\textsuperscript{42, 89, 102, 103}.

The adsorbed amounts can also be calculated according to the de Feijter formula (paper III)\textsuperscript{143}:

$$\Gamma = df / dn / dc \quad \text{Eq. 5}$$

An assumption of linearity of the increment in refractive index with concentration is the basis of this equation. A dn/dc value of 0.16 ml/g\textsuperscript{20, 82, 83} was used, based on the characteristics of the mucin.

Comparison between the models of Cuypers and de Feijter, respectively, has shown quantitatively the same results\textsuperscript{144}.

A drawback when using ellipsometry is that no information on the composition of the adsorbed film is obtained. Nevertheless, for single protein solutions the results can be interpreted in terms of e.g. orientation of adsorbed molecules. It should be kept in mind that the thickness obtained from
ellipsometry measurements assumes a homogeneous film with a mean refractive index. This results in a film thickness that is averaged over the measured area of the film, which may not necessarily describe the real situation. Further, surfaces need to be planar, smooth and reflecting, and the solution has to be transparent to avoid too much light scattering, which otherwise decreases the sensitivity of the measurement.

**Mass transport analysis**

The initial adsorption kinetics of the cationic proteins (papers I and II) was investigated to elucidate if the adsorption was mass transport controlled. To this end, the model by Trurnit was employed. The model relies on a stirred system where the mass transport to the solid/liquid interface is determined by diffusion over an unstirred layer closest to the surface. The thickness of this unstirred layer was in the present investigations estimated to be 20 µm. Trurnit’s model further assumes an irreversibly adsorbed layer where the time to create a constant concentration gradient in the unstirred layer is negligible. Conditional on the above-mentioned criteria, the initial adsorbed amounts versus time, for a diffusion controlled system are:

\[
\Gamma = \frac{DC_0t}{\delta} \tag{Eq. 6}
\]

where \(\Gamma\) is the adsorbed amount (mg×m⁻²), \(D\) the diffusion coefficient (m²×s⁻¹), \(C_0\) the bulk concentration (mg×m⁻³), \(\delta\) the thickness of the unstirred layer (m) and \(t\) is the time (s).

**Atomic force microscopy (AFM)**

AFM was employed to investigate the structure of the adsorbed protein films, as described in paper III. For these experiments, AFM imaging was performed to acquire images at different stages in the multilayer formation. Values on surface roughness, size, shape, as well as distribution of adsorbed components can be obtained, with a resolution in the z-direction down to the nanometre or Ångström scale.

Briefly, AFM imaging is based on scanning a surface using a cantilever with a very sharp tip. Interactions between the sample/surface and the tip will cause the cantilever to bend. A laser beam is reflected on the cantilever and will
register changes in the cantilever position. These changes are then recorded by the photodetector and converted to a 3D reconstruction of the sample topography. In contact mode, which was used here, the interaction between the tip and surface is kept constant by moving the sample in the z-direction during scanning.

AFM is a powerful technique and has the advantage, in comparison to other similar techniques such as transmission electron microscopy, that it only requires minimal sample preparation and images can be acquired in aqueous solution at ambient temperatures, simulating physiological conditions. However, adsorbed protein films, which are of a soft nature, can be deformed during scanning, resulting in altered structures that do not resemble the actual film morphology. Therefore, in the experiments presented here, the surface load was fixed to balance the electrostatic repulsive barrier\textsuperscript{248}, to minimize such alterations. The experiments were performed in a liquid cell, and the images were obtained after rinsing the liquid cell with buffer, to reduce interference due to e.g. viscoelasticity from components in the ambient solution.

The instrument used was a scanning probe microscope from Veeco (Picoforce multimode SPM with a Nanoscope IV control unit). Silicon nitride tips with a cantilever spring constant of ≤0.32 N/m from Veeco, type DNP, were used.

**Total internal reflectance fluorescence (TIRF)**

TIRF is a technique that detects fluorescence from components adsorbed at, or close to, an interface. The fluorescence may be emitted from intrinsically fluorescent amino acids or, as described in paper IV, from fluorescent groups covalently bound to a protein.

The basic principle of TIRF is the excitation of fluorescent molecules by an evanescent wave that forms in the liquid phase close to the interface. The evanescent wave is a result from superposition of incident and reflected beams upon total reflection at the interface between two media with different optical properties. The evanescent wave decays exponentially normal to the interface on the far side of the optically rarer medium (the protein solution), thereby confining the excitation to the interfacial region. In the experimental set-up used here, the penetration depth, which is the distance over which the evanescent wave amplitude decreases to $e^{-1}$ of its initial value, was estimated to be 200 nm. A detailed description of the instrument used is given by Lassen.
and Malmsten\textsuperscript{46}. Briefly, it consist of a 488 nm argon ion laser (model 161 B, Spectra physics, USA) as a light source, a flow cell, and the detection part of the instrument consisted of a monochromator (Model H20 UV, Jobin Yvon, France), a photo-multiplier tube (Model R 298, Hamamatsu, Japan) and a photocounter (Model SR 400, Stanford Research System, USA).

TIRF is a technique that records adsorption in a time-resolved manner, similar to ellipsometry. It is a good complement to ellipsometry, as both the total adsorbed amount as well as the fraction of specific components in the adsorbed film can be elucidated by these techniques. Due to factors such as quenching, uneven probe distribution across the adsorbed layer, and local pH fluctuations, quantification of the adsorbed amounts is not straightforward, and therefore the results obtained by TIRF (paper IV) was analysed based on relative changes in fluorescence.

As for ellipsometry, TIRF requires a transparent solution, and the surfaces used for TIRF needs to be smooth, planar and transparent, to allow total reflection at the interface between the surface and the protein solution.

For the TIRF experiments, PRP-1 and statherin were fluorescently labelled with fluorescein-5-isothiocyanate (FITC). The average molar ratio of FITC to protein was <0.15 mol FITC/mol protein. The low labelling densities minimizes the effect of the fluorophore on the protein structure and decreases risks of quenching or changes in adsorption behaviour. However, the signal to noise ratio of the measurements may be lower.

**Experimental outline**

Figure 6 shows the general experimental outline for the experiments described in papers I-IV. Time intervals are given in the respective papers.
Figure 6. Experimental outline. n symbolizes number of times the procedure was repeated (n=1-4, papers III and IV).

**Pellicle protein composition**

*In vivo pellicle collection and in vitro validation*

The *in vivo* pellicle collections were performed by rubbing tooth surfaces with fibre pellets (Quick-sticks™, Dentonova AB, Huddinge, Sweden) soaked in different concentrations of SDS (2%, 0.5%, 0.25% and 0.1%(w/v), respectively). The collection, as described in detail in paper V, was based on a previously reported procedure, and was performed from one donor considered to be in good oral and general health. The teeth were isolated during pumicing, cleaning and collection; contact with gingiva as well as the crevical third and approximal areas of the teeth was avoided. After the collection with 3 fibre pellets soaked in SDS solution, one dry fibre pellet was also used.

For the *in vitro* validation, HWS was collected as described above, from the same donor as the *in vivo* pellicle. Extracted teeth were used for the validation trial. Each tooth was treated separately during the validation test, except
during the *in vitro* pellicle formation. A detailed description of the validation is given in paper V. The *in vitro* pellicle collection was performed by using three fibre pellets, soaked in 0.5% SDS (w/v) and one dry fibre pellet per 3 teeth (both buccal and palatal/lingual sides were collected).

**Investigation of *in vitro* pellicles from different oral surfaces**

Figure 7 shows the procedure of the *in vitro* pellicle formation on human enamel, titanium and PMMA, respectively, which is described in detail in paper VI.

![Procedure for in vitro pellicle investigation.](image)

The experiments were performed at room temperature. The substrates in Figure 7 symbolise the respective type of material, while the empty container symbolizes the HWS control with no substrate submerged. HWS remaining after *in vitro* pellicle formation was stored at -20 °C until subjected to 2-DE.

**Gel electrophoresis and staining**

Gel electrophoresis is a technique that separates proteins on account of some specific characteristics, usually the molecular weight or charge. The proteins move at different velocities in an electric field, based on e.g. their molecular mass. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels are frequently used, where SDS applies negative charge to the proteins, yielding an almost uniform charge to mass ratio for all proteins. As a result, the migration in the gel is assumed to only depend on the size of the proteins.

Combining isoelectric focusing (IEF) with SDS-PAGE results in two-dimensional gel electrophoresis (2-DE). IEF, which is performed prior to SDS-PAGE, separates proteins based on their isoelectric point (pI); proteins migrate
in the immobilized pH gradient of the gel until they are uncharged, and are thus positioned at a pH corresponding to their respective pI’s.

For the first dimension (IEF), a relatively wide pH gradient (pH 3-10) was used, to be able to visualize a large fraction of the complex protein composition of saliva and pellicle. The gel used in the second dimension (SDS-PAGE based on 10-14% Tris/Tricine) was prepared based on a previously described technique, to gain good separation of the low molecular weight composition ($\leq$approximately 70 kDa). Specifics of the 2-DE can be found in the literature. It has been shown that the majority of salivary proteins have pI’s between 4 and 8 and molecular weights (Mw) below 120 kDa (with 45% having Mw $<40$ kDa), justifying the choice of pH and size range. An amount of 7 µg of protein was applied to each IPG-gel strip. For the validation samples (paper V), 12% Ready Gel® Precast SDS-PAGE gels (Bio-Rad Laboratories, Sundbyberg, Sweden) were used. The protein size range of these gels is approximately $\leq$250 kDa.

Silver staining was used to detect the proteins in the gels and was performed as described by Heukeshoven et al. The proteins in the gel bind silver ions, which then are reduced to elementary silver, resulting in protein visualization. This technique can detect proteins down to about 1 ng of protein. However, there may be some discrepancies in staining between different proteins, due to the preferential interaction of silver ions with some specific amino acids. Furthermore, the quantitative range is rather limited; highly abundant proteins often appear with a yellow centre, which 2D quantification software may interpret as low abundance due to the light colour. Therefore, for the 2D quantification performed using Delta2D from Decodon GmbH (Greifswald, Germany), the spots analysed were chosen based on clear and distinct staining, as well as avoidance of highly abundant spots.

To increase the staining sensitivity, the in vivo pellicle and in vitro validation samples were stained with Coomassie Brilliant Blue (CBB; Sigma St Louis, MO, USA, No B-2025), prior to silver staining.

**Protein content analysis**

The Bio-Rad RC/DC protein assay (Bio-Rad, Sundbyberg, Sweden) was used to estimate the protein concentration in the salivary samples. This is a colorimetric assay (based on the Lowry protocol), where colour develops as a result of the protein concentration. According to the manufacturer, the assay is particularly responsive to some amino acids, which could result in colour
differences between proteins. However, as other studies have applied these types of techniques for similar purposes\textsuperscript{157, 158}, it was considered as a reliable, complementary technique to the 2-DE analysis. The linear range of the chosen RC/DC protein assay using BSA as a standard is reported to be 0.2-1.5 mg/ml. The obtained values of the pellicle samples (paper V) were slightly below this range, but were still considered as reliable for inter-sample comparison.
RESULTS AND DISCUSSION

This part of the thesis has been divided into three sections; adsorption of single components, adsorption of several components, and finally adsorption from complex solutions. The main results are presented in this context, whereas the reader is referred to the respective papers for further details.

In the first section (papers I and II), the adsorption behaviour of the cationic proteins lactoferrin, lactoperoxidase, lysozyme and histatin 5, respectively, is addressed. In the second section (papers III and IV) sequential alternating adsorption experiments are discussed as well as the layer-by-layer film formation with anionic MUC5B and cationic lactoperoxidase. The interaction of MUC5B as well as HWS with the anionic pellicle precursor proteins PRP-1 and statherin, respectively, at the solid/liquid interface is also considered. The influence of the anionic surfactant SDS on the protein films is investigated in all systems. In the last section, the complex film formation of HWS with respect to in vivo and in vitro pellicles is addressed. The compositional characteristics of pellicles formed on enamel as well as on titanium and PMMA, with respect to low molecular weight proteins with pI’s 3-10, are described.

The majority of experiments is performed using model systems, under very strict and controlled conditions, and may therefore not be directly applicable to the in vivo situation. These types of investigations are still of relevance in order to elucidate the underlying forces responsible for the salivary film formation. An attempt to describe the clinical relevance of the findings is included at the end of each section.
Single component systems (papers I and II)

Adsorption isotherms

For all investigated proteins, a plateau seemed to be reached regarding adsorbed amounts within the concentration ranges investigated. The adsorption appeared to be driven by electrostatic interactions on the hydrophilic, negatively charged substrate, whereas on the hydrophobized silica, hydrophobic interactions were of importance. The protein characteristics, such as size and charge/hydrophobicity, influenced the shape of the isotherm, and these factors are discussed below. The adsorption isotherms are shown in Figure 8, and are plotted both as nmol m$^{-2}$ vs. µM (Figures 8a-b), which excludes the role of the molecular weight, as well as mg m$^{-2}$ vs. µg ml$^{-1}$ (Figures 8c-d). From these curves, it is obvious that the molecular weight has a large influence on the appearance of the adsorption isotherms. The isotherm of histatin 5 is a clear example of this. It can be seen in Figures 8a-b, that histatin 5 has the highest adsorption affinity (i.e. number of molecules on the surface in relation to the bulk) on both types of surfaces. The adsorbed mass, however, is below monolayer coverage, indicating e.g. intermolecular repulsion within the adsorbed layer. In Figures 8c-d it can be seen that the adsorption of histatin 5 is not as pronounced as that of lactoferrin and lactoperoxidase, and both these proteins adsorb in amounts corresponding to monolayer coverage or more (i.e. adsorbed molecules close-packed on the surface). In the following discussions, the units of mg m$^{-2}$ vs. µg ml$^{-1}$ will be used, and adsorbed amounts will be compared with monolayer coverage.

The adsorbed amounts of lactoferrin and lactoperoxidase were similar and generally higher on hydrophilic silica compared with on hydrophobized silica (Figure 8). The hydrophilic and hydrophobized silica surface have been shown to have a similar zeta potential values. Therefore, it seems that the methyl groups on the hydrophobized surface decrease the adsorption of lactoferrin and lactoperoxidase, possibly due to protein unfolding/conformational changes at the surface. The results presented here are in agreement with previous adsorption studies on lactoferrin and lactoperoxidase. The adsorbed amounts at plateau values indicate a layer composed of both side-on and end-on adsorbed molecules for lactoferrin on both types of surfaces, as well as for lactoperoxidase on hydrophobized silica (Figure 8 and Table 1). On hydrophilic silica, lactoperoxidase is adsorbed in amounts corresponding to an end-on monolayer or more. Calculations of the surface area per molecule of lactoperoxidase (paper II) indicated that the adsorbed molecules
either had a lower surface area per molecule compared with an end-on monolayer (i.e. altered structure), or that a partial bilayer was formed.

Comparing the isotherms of lactoferrin and lactoperoxidase, lactoferrin had a higher adsorption affinity for hydrophilic silica, whereas lactoperoxidase had a higher affinity for the hydrophobized surface (Figure 8; paper I Table 2). This indicates that electrostatic interactions have a more prominent role in the adsorption of lactoferrin, whereas hydrophobic interactions are more important for lactoperoxidase adsorption. The higher aliphatic index (Table 1) as well as the lower charge of lactoperoxidase compared with lactoferrin (which is known to have a high density of charges present at the surface\(^{10}\)) may explain this behaviour.

**Figure 8.** Mean plateau values of adsorbed amounts after 120 min of adsorption vs. protein concentration in solution. Lactoferrin (blue), lactoperoxidase (green), lysozyme (black) and histatin 5 (red).
Lysozyme had a higher affinity for the hydrophobized substrate compared to the hydrophilic substrate, which may be justified by the shell hydrophobicity of lysozyme, as 41% of the water accessible area has been reported to be occupied by nonpolar groups. An adsorbed amount of 1.7 mg m$^{-2}$ was obtained on both hydrophilic and hydrophobized substrates for 200 µg ml$^{-1}$ of lysozyme (paper I, Table 2), which corresponds to a side-on monolayer (Table 1).

The adsorption isotherm of histatin 5 was similar on the two types of surfaces (Figure 8). Due to the low content of hydrophobic side chains and high fraction of positively charged side chains, histatin 5 may be viewed as a small, positively charged polyelectrolyte, and as a result, the adsorption appeared to be controlled by electrostatics on both types of substrates. The adsorbed amount corresponds to a side-on monolayer of stretched molecules (0.9 mg m$^{-2}$, paper I, Table 1). However, due to the large positive charge of Histatin 5 (+5mol/mol, Table 1), and in spite of the relatively high ionic strength of the solution (80 mM, i.e. $\kappa = 15$ Å), intermolecular repulsion within the adsorbed film is likely and hence the adsorbed film could consist of coiled molecules, with a surface coverage below monolayer coverage.

Lysozyme and histatin 5 were adsorbed in lower amounts (with respect to fraction of monolayer) on both types of substrates compared to lactoferrin and lactoperoxidase (Figure 8 and Table 1). The experiments were performed in PBS buffer at pH 7.0. Both lactoferrin and lactoperoxidase have a lower net charge at this pH compared to lysozyme and histatin 5, and hence it is not surprising that these larger proteins adsorb in amounts corresponding to monolayer coverage or even more. A lower net charge would result in decreased intermolecular repulsion within the adsorbed layer and hence a more efficient packing of adsorbed molecules.

**SDS interactions with adsorbed protein films**

The results of SDS elution on hydrophilic silica indicated that the protein desorption occurred through formation of SDS-protein complexes (Figure 3a), as SDS is known not to adsorb to hydrophilic silica. On hydrophobized substrates, desorption probably took place both by the formation of soluble complexes as well as by replacement of adsorbed proteins by SDS (Figures 3a-b). Mainly hydrophobic interactions between the adsorbed proteins and SDS are expected.
On hydrophilic silica, a large fraction of the adsorbed films were removed upon addition of a SDS solution, for all investigated proteins (for examples see Table 3). A small amount of adsorbed proteins remained on the surface for all proteins (Table 3 and paper I, Table 2), indicating that a minor amount was strongly attached to the surface, due to e.g. conformational changes.

On hydrophobized surfaces, a smaller fraction of the adsorbed film was eluted by SDS compared to hydrophilic silica (Table 3), indicative of stronger binding to this surface type.

Table 3. Adsorbed amounts at isotherm plateaus for the respective proteins, at different stages in the adsorption experiments. R= buffer rinsing. Percentage of side-on monolayer coverage is given within parenthesis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrophilic substrates</th>
<th>Hydrophobized substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before SDS (mg m⁻²)</td>
<td>Before SDS+R (mg m⁻²)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>3.7 (245%)</td>
<td>2.5 (167%)</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>3.2 (152%)</td>
<td>2.5 (119%)</td>
</tr>
<tr>
<td>100 µg ml⁻¹</td>
<td>0.9 (50%)</td>
<td>1.7 (94%)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.6 (20%)</td>
<td>0.5 (17%)</td>
</tr>
<tr>
<td>10 µg ml⁻³ Histatin</td>
<td>0.6 (20%)</td>
<td>0.5 (17%)</td>
</tr>
</tbody>
</table>

Comparing the results obtained for the different proteins, the fraction remaining after SDS assisted desorption, can be seen as a measure of the adsorption strength. Lactoperoxidase seems to be the protein most difficult to remove by SDS on hydrophobic surfaces, which also may be expected considering its high aliphatic index (Table 1). Histatin 5 was also difficult to remove with SDS, possibly due to the strong electrostatic interaction with the negatively charged surfaces. Note that the data for histatin 5 elutability may be inaccurate due to the limited adsorbed amounts prior to SDS elution (see percentage of monolayer in Table 3).
Initial adsorption kinetics of lactoperoxidase and histatin 5 in relation to adsorption from HWS

The initial adsorption data was compared with theoretical predictions of initial adsorption rates (Eq. 6), to get an estimate of at what stage the adsorption is shifted from being controlled by mass transport to being limited by other factors, such as e.g. intermolecular interactions on the surface.

![Graph showing adsorption kinetics](image)

**Figure 9.** Initial adsorption of lactoperoxidase (a), histatin 5 (b) and HWS (c) to hydrophilic (◇) and hydrophobized (▼) silica, compared with the calculated mass transport controlled adsorption rate (Eq. 6) of lactoperoxidase (green line) and histatin 5 (red line), respectively.

The results showed that the adsorption of lactoperoxidase was mass transport controlled during the first 1.5 min of adsorption on both types of surfaces (Figure 9a). For histatin 5, adsorption during the first 30 sec was
controlled by mass transport (Figure 9b). The results further show that both proteins deviated from the calculated mass transport controlled adsorption rates at a surface coverage below 50% of that of a monolayer (arrows in Figures 9a-b). This indicates packing restrictions at the surface due to e.g. lateral repulsive forces between adsorbed proteins. When compared with the initial adsorption from HWS, taking into account the concentrations of the proteins in the secretion (Table 1), it was shown that proteins/peptides of similar size as histatin 5 were involved during the early stages of HWS adsorption. On hydrophobized silica, the adsorption from HWS was faster compared to the diffusion rates of both lactoperoxidase and histatin 5, indicating that peptides smaller than histatin 5, or similarly sized peptides present at higher concentrations, are involved in the initial adsorption.

Relevance for the clinical situation

Lactoferrin, lactoperoxidase, lysozyme and histatin 5 adsorb in different amounts to the two types of substrates investigated. With respect to the antimicrobial functions of these proteins, the depot of such components may be different depending on the underlying substrate characteristics, which could result in shifts in the plaque composition. Studies have shown that the initial plaque formation is influenced by the substrate characteristics\textsuperscript{160-163}, which may be a result of differences in adsorbed amounts and proportion of salivary components on different surfaces.

Histatin 5 has been shown to have a decreased antifungal ability when adsorbed to surfaces, but once desorbed it regains its activity\textsuperscript{60}. An IC\textsubscript{50} of 1.4 µM has been reported for histatin 5\textsuperscript{164}, and this concentration would result in a maximum adsorbed amount of 0.5 mg m\textsuperscript{-2} on a given surface (Figure 8). If a denture surface area of 100 cm\textsuperscript{2} is assumed, a maximum amount of about 5 µg of histatin 5 could be desorbed. Based on a residual saliva volume of 0.8 ml in the mouth and an unstimulated salivary flow rate of 0.3 ml/min\textsuperscript{1}, the desorbed histatin 5 amount would not be sufficient to reach the IC\textsubscript{50} concentration. Therefore, the incorporation of histatin 5 in multilayered structures, or as components in mouth rinses could be a way to increase the supply of histatin 5 in the mouth.

The interaction with SDS may decrease the amount of active components on oral surfaces. On the other hand, SDS may induce an increased release of adsorbed proteins. In this context it should be realized that the SDS-protein complex in solution may not be as effective as the pure protein. It may be
expected that the denatured structure of the protein after SDS elution would reduce the activity.

**Systems with several components (papers III and IV)**

Layer-by-layer adsorption of mucin and lactoperoxidase

Large differences in the build-up of multilayered films were observed between the particular mucin (MUC5B and BSM, see below) and the specific cationic protein, respectively (paper III, Figure 2). MUC5B and lactoperoxidase was the most successful combination. It may be speculated that the closely matched charge densities between lactoperoxidase and the glycosylated domains of MUC5B is the reason why this combination was most successful in multilayer constructions.

The build-up of four consecutive bilayers of MUC5B and lactoperoxidase on both types of silica substrates are shown in Figure 10.

![Figure 10. Adsorbed amount vs. number of adsorption cycles of MUC5B/lactoperoxidase on hydrophilic silica (◊) and hydrophobized silica (♦), and of BSM/ lactoperoxidase on hydrophilic silica in PBS (Δ) and in 10 mM phosphate buffer + 1 mM CaCl₂ (○). Lines are inserted to guide the eye.](image)

As can be seen, there was a linear increase in adsorbed amount following the first adsorption of MUC5B on both types of substrate. The increase was somewhat more pronounced on hydrophilic silica, particularly with an increasing number of adsorption cycles, which indicates that the substrate characteristics are of importance, even after the completion of several
adsorption cycles. This is in line with other observations which showed that multilayer formation is substrate dependent\textsuperscript{82,83}.

The AFM experiments showed that MUC5B was adsorbed as islands, and the following lactoperoxidase adsorption seemed to take place both to the bare hydrophilic surface as well as by interacting with MUC5B, thereby changing the structure to a relatively smooth surface film (paper III, Figure 4).

The alternating increase and decrease in adsorbed amount upon lactoperoxidase and MUC5B adsorption, respectively (after the first adsorption of MUC5B; Figure 10), may be regarded as a process of adsorption and re-dissolution during film formation. The proposed mechanism for this behaviour is described in paper III. Briefly, it can be explained from the stability of the corresponding complexes, so called coacervates, formed in solution\textsuperscript{165}.

The possibility of constructing multilayers with lactoperoxidase and the commercially available mucin BSM was also investigated. The experiments, performed at similar concentrations as for MUC5B/lactoperoxidase and in PBS at 37°C, showed that layer-by-layer formation with these components was very limited (Figure 10). The build-up was enhanced in 10 mM phosphate buffer supplemented with 1 mM CaCl\textsubscript{2}, which might be due to effects on the electrostatic interactions.

**SDS interactions with adsorbed multilayered films**

A larger fraction of the adsorbed film was eluted from the hydrophilic surfaces compared to the hydrophobized surfaces. Further, the number of adsorption cycles influenced the SDS elutability on hydrophobized silica.

Table 4. Adsorbed amounts at different stages in the adsorption of MUC5B/lactoperoxidase, after 2 and 4 adsorption cycles respectively. R= buffer rinsing.

<table>
<thead>
<tr>
<th></th>
<th>Hydrophilic substrates</th>
<th></th>
<th>Hydrophobized substrates</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before SDS (mg m\textsuperscript{-2})</td>
<td>After SDS (mg m\textsuperscript{-2})</td>
<td>% eluted</td>
<td>Before SDS (mg m\textsuperscript{-2})</td>
</tr>
<tr>
<td>MUC5B/lactoperoxidase (2 bilayers)</td>
<td>4.5</td>
<td>0.7</td>
<td>85</td>
<td>7.7</td>
</tr>
<tr>
<td>MUC5B/lactoperoxidase (4 bilayers)</td>
<td>10.0</td>
<td>1.4</td>
<td>86</td>
<td>9.6</td>
</tr>
</tbody>
</table>
The observed stronger adsorption to hydrophobized substrates (Table 4) are in line with general observations. The film on hydrophobic silica was more compact (similar thickness but higher adsorbed mass, i.e. higher density, after two adsorption cycles; paper III, Figure 1) compared to the film on hydrophilic silica. A denser film could limit SDS interactions with the adsorbed components and hence result in decreased film elutability.

Sequential adsorption of salivary components

The sequential adsorption of PRP-1 and statherin followed by MUC5B and HWS, respectively, was influenced by the substrate characteristics, which may be due to the pre-adsorbed amount and orientation of PRP-1 and statherin, respectively. The pre-adsorbed PRP-1 on hydrophobized silica, approximately 60% of a monolayer (Table 1), decreased the adsorption from both HWS and MUC5B, respectively, indicating decreased attraction between the PRP-1 film and HWS/MUC5B components compared to the adsorption of these components on the bare substrate (Table 5). The lower adsorbed amounts of PRP-1 on hydrophilic surfaces (25% of a monolayer) did not influence the subsequent adsorption from HWS, which is in line with previous studies.

Possible exchange phenomena

As both PRP-1 and statherin are small proteins, an exchange of these components with MUC5B and also with components in HWS was expected. However, the results from the TIRF experiments indicated only minor exchange in all of the investigated systems. The largest exchange observed was for PRP-1 in sequence with HWS on hydrophilic substrates (Figure 11). These results corroborate the general trend that proteins are more readily displaced from hydrophilic substrates, when compared to hydrophobized surfaces. Overall, the limited exchange observed in these model systems indicates that an exchange similar to the “Vroman effect” in blood is not as pronounced in saliva.
Figure 11. Relative fluorescence (after buffer rinsing) of FITC-labelled PRP-1 in sequence with 10% HWS on hydrophilic (○) and hydrophobized (●) substrates. Lines are inserted to guide the eye.

**SDS interactions with adsorbed mixed films**

The effect of SDS elution was investigated to gain insight into possible differences between adsorbed mixed films and the individual components separately.

**Table 5. Adsorbed amounts at different stages in the adsorption of displayed proteins.** R= buffer rinsing, *no data (paper IV).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrophilic substrates</th>
<th>Hydrophobized substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before SDS</td>
<td>After SDS+R</td>
</tr>
<tr>
<td>PRP-1</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>HWS</td>
<td>2.8</td>
<td>0.1</td>
</tr>
<tr>
<td>MUC5B</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>PRP-1/HWS</td>
<td>3.3</td>
<td>0.4</td>
</tr>
<tr>
<td>PRP-1/MUC5B</td>
<td>1.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

A decreased elutability of the PRP-1/MUC5B mixed films on both types of substrates indicated complex formation between the components, which is
also known to occur in solution. The resulting film was more resistant to SDS elution. It has previously been shown that high molecular weight fractions of saliva are harder to remove by surfactant rinsing, compared to low molecular weight fractions. The results presented here are in accordance with those findings, and further show that high molecular weight components, such as MUC5B, may protect other proteins from surfactant elution. The protection may be due to the higher mass of the PRP-1/MUC5B complexes and hence more surface anchoring sites, and/or cross-linking within the adsorbed film.

The combination of PRP-1 and HWS on hydrophilic surfaces also seemed to indicate complex formation resulting in a decrease in PRP-1 elution, possibly due to interactions with MUC5B in HWS. On hydrophobized substrates, the PRP-1/HWS film was more readily removed compared to the individual components (Table 5), which could be a result of a lower film density (paper IV, Tables 1-2), resulting in a more open structure that is accessible for SDS interaction.

Relevance for the clinical situation

Adsorbed multilayered structures have the potential to i) increase the incorporation of active components in the film, ii) influence the barrier function of the film, e.g. lubrication and iii) influence the cohesion of the film (in terms of interactions with surfactants; papers III and IV).

Multilayered structures of MUC5B and lactoperoxidase may have augmented antimicrobial properties; recent studies indicate that the activity of lactoperoxidase may be enhanced when adsorbed to pre-adsorbed MUC5B compared to adsorption to a clean surface. This could be of importance during the microbial biofilm formation, speculating that the concentration of inhibitory thiocyanate derivatives produced by lactoperoxidase is increased and the inhibitory components are released close to the microbes in the plaque, thereby increasing the efficiency in inhibiting e.g. growth and metabolism of susceptible species. Analogously, increased enamel de- and remineralisation processes and lubrication may be anticipated for PRP-1 in PRP-1/MUC5B films.

The observed increase in resilience to SDS elution in some of the experiments could indicate that the proteins incorporated in the film may be protected from surfactants present in oral care products. The effect is dependent on the appropriate material characteristics, and thus may be different on different surfaces present in the mouth.
Complex systems (papers V and VI)

Elution of enamel pellicles

By means of combining mechanical (rubbing) and chemical (0.5% SDS) action it was possible to remove the pellicle and further separate the components with 2-DE without any demanding sample preparation.

Figure 12. 2-DE of in vivo pellicles collected with different concentrations of SDS: 0.25 % SDS (A), 0.5 % SDS (B) and 2 % SDS (C).
As shown in Figure 12, the collection of pellicle material with 2% SDS showed a high degree of interference from horizontal streaking in 2-DE, a result of too high SDS concentration in the rehydration solution used for the IPG gel. As further shown in Figure 12, the collection with 0.25% SDS, i.e. at the cmc in water, was insufficient as fewer protein spots were detected. The data from the protein content analysis confirmed these results (paper V, Table 1).

For protein elution from surfaces, a concentration close to the surfactant cmc is usually required for maximum protein desorption. However, in the present study a concentration of 2×cmc had to be applied for total pellicle collection. Based on the volume per fibre pellet and reported amounts of pellicle proteins given the labial tooth surface, a value of 1.2 g SDS per g protein was obtained when using a 0.25% SDS solution. A wide range of proteins have been reported to bind a maximum of 1.4 g SDS/g protein, indicating that a concentration of 0.25% SDS might be insufficient for complete protein-SDS complex formation.

The validation test indicated that rubbing with fibre pellets soaked in 0.5% SDS enabled the removal of the complete pellicle from the tooth enamel surface. After the in vitro pellicle had been collected, the tooth surfaces were rubbed twice with PVDF membrane filters soaked in HCl followed by PVDF membranes soaked in water, and the results of the validation samples (1-DE and protein assay) showed no indications of any proteinaceous material remaining on the surfaces (paper V, Figure 2 and Table 1). The results show that the concentration of elution agent is of importance. As previous ellipsometric studies showed that rinsing with SDS solutions did not remove the complete salivary film, it appears as if the combination of chemical and mechanical treatment is required to remove and enable the collection of the salivary film.

Previous studies have indicated that mechanical rubbing in combination with 2% SDS solution was insufficient to completely remove the in situ formed pellicle on bovine enamel slabs. This indicates that the technique is sensitive to small procedure differences, such as e.g. pre-treatment and/or cleaning of the surface, as well as type of surface and type of fibre pellet used.

For the validation tests, 12% SDS-PAGE gels were used, which have a larger detectable protein size (≤250 kDa) span compared to the 2-DE gels (≤70 kDa), to enable the detection of higher molecular weight components possibly remaining. Furthermore, the protein assay, used as a complement, would also
disclose the presence of any size of proteins. Although it was indicated that the entire pellicle was collected with the technique described in paper V, the elution from the fibre pellets was incomplete, as shown by placing a fibre pellet directly onto the SDS-PAGE gel (paper V, Figure 3). Hence, the elution procedure needs to be optimized. Heating of the fibre pellets prior to centrifugation, as performed in other studies\textsuperscript{61, 70, 157} may increase the amount of proteins eluted; heating is known to increase the solubilization of proteins, and thus likely the release of proteins from the fibre pellets.

**The composition of *in vitro* pellicles is substrate influenced**

We also used 2-DE to demonstrate differences in the low molecular weight region of pellicles formed on human enamel, titanium and PMMA surfaces, respectively (Figure 13). Large differences were observed between titanium and enamel in the pI range 5-7. In addition, proteins with pI ~9 and molecular weights below 30 kDa, seem to be adsorbed to two or more of the investigated substrates. This supports previous findings on the role of cationic salivary proteins in the adsorption process\textsuperscript{52}.

![Figure 13. Silver stained 2-DE of the HWS control. The proteins that are absent in saliva after incubation with the substrates (and thus assumed to be incorporated in the pellicle) are illustrated by \ldots \ldots; enamel, \ldots; titanium, and \ldots: PMMA.](image)

One should keep in mind that only 7 µg protein per gel was applied, thus only the major differences in protein pattern are detected. Therefore, the proteins encircled in Figure 13 (i.e. the ones that have disappeared from the
respective gels and thus are incorporated in the pellicles) may be considered as major components in the pellicle. However, their relative abundance in the complete composition is still not known as no analysis of the pellicle proteins with higher molecular weight was performed.

To get a better estimate of the composition of the different pellicles, the 2-DE gels were also analysed with the 2-DE image software Delta2D from Decodon GmbH (Greifswald, Germany). As shown in Figure 14, there are major differences between the saliva samples left after incubation with the different surfaces. The software analysis was based on silver stained 2-DE gels, which has a limited dynamic range (page 44). Therefore the results shown in Figure 14 should be interpreted with some caution. The ten proteins selected for deeper analysis (paper VI, Figure 1 and Table 2) were chosen to avoid factors limiting the dynamic range of the silver staining technique.

![Figure 14. 2-DE of the HWS control. Spots coloured are proteins present in reduced amounts in the saliva samples left after incubation with enamel (green), titanium (yellow), PMMA (blue). The red colour symbolizes proteins that are present in reduced amount in two or more of the depleted saliva samples.](image)

This study shows that there are compositional differences in pellicles, reflecting the substrate characteristics; circumstances also supported by previous studies\(^{40, 63, 69, 70, 157, 171-173}\). Other studies have shown that the substrate characteristics to some extent are transferred to the protein-bacteria interface, and that different materials promote selective adherence during early plaque formation\(^{160-163}\), which could partly be a consequence of the differences in
pellicle compositions. Although some studies have shown that the pellicle formation evens out differences in substrate characteristics\textsuperscript{174, 175}, this does not rule out but rather supports the possibility of different compositions of the pellicles formed on different substrates.

Relevance for the clinical situation

As the results presented in paper V indicated a complete removal of the pellicle, it would be a realistic goal in the near future to be able to characterize the complete pellicle composition, also regarding larger pellicle constituents, and also to quantify individual components. This could be of importance in e.g. diagnostics, where the specific identification of “marker proteins” (i.e. proteins known to be associated with specific conditions) may result in identification of diseases at early stages. Patients with e.g. Sjögrens syndrome, 22q11 deletion syndrome, or cancer have been shown to have an altered salivary composition\textsuperscript{176-179}, which could be of significance for the pellicle formation, and thus of relevance for the oral health of these patients. From this perspective it would be interesting to study pellicles from saliva of patients suffering from objective as well as subjective feeling of dry mouth, as it seems that it is the quality of the film and not the quantity of saliva that determines the feeling of dry mouth. To be able to develop products aimed at relieving these patients from their oral handicap, e.g. diminished lubrication and escalating caries disease, it is of importance to understand the influence of the film quality on oral health.
The present thesis is an account of investigations on salivary films of different degrees of complexity, to elucidate the influence of diverse surface-protein and protein-protein interactions on surface adsorption processes. It was shown that the substrate characteristics influenced the surface film formation for all films studied. Both quantitative and qualitative differences could be observed. The adsorption behaviour of the cationic proteins investigated was dominated by electrostatic interactions when adsorbed to negatively charged silica surfaces. For the construction of layer-by-layer adsorbed films with MUC5B and lactoperoxidase, the substrate characteristics were found to affect the build-up process. Furthermore, the film density and number of adsorption cycles were found to influence the elutability with respect to SDS. In the case of sequential adsorption of PRP-1 and MUC5B, only a small exchange of pre-adsorbed PRP-1 by MUC5B could be observed, however, the film resilience to SDS elution was increased for the mixed film indicating complex formation between adsorbed components. The substrate characteristics had an influence on how these complexes form, which indicates that the adsorbed amounts and the orientation of adsorbed components determine the interactions with other constituents. It was also shown that small proteins, in the size range of histatin 5, were involved in the initial adsorption from HWS, which also was indicated in the studies of pellicle formation on different surfaces. This finding is supported by previous results regarding a large amount of small proteins/peptides in saliva, resulting from e.g. proteolysis.

As the development of oral care products containing surfactants that are less harmful compared to SDS is accelerating, it would be of significance to
investigate the influence of such surfactants on salivary films in a manner similar to the work presented in this thesis. Also, considering the sequential (alternating) adsorption experiments, it would be interesting to try to compose multilayered structures containing more than two components to be able to incorporate additional functions in the films, to target several conditions that may occur in the oral cavity. In this context, it is of course also of importance to study the influence of *in vivo* conditions, e.g. proteolytic degradation, as well as the effect of using different substrates.

Considering the complex build-up behaviour of the pellicle, the implication of the differences in pellicle compositions between different dental materials on the initial plaque formation would be an interesting aspect, which already to some extent has been addressed in the literature. The identification and specifically the quantification of the pellicle components are of importance for studies on the influence of pellicle composition on plaque formation and oral disease progression.
Munhålan är en mycket komplex miljö som vi inte reflekterar över så ofta. Många viktiga funktioner i munnen kan vi till stor del tacka saliven för. Saliv består av 99% vatten, och den resterande delen består av en mångfald av komponenter, såsom proteiner, kolhydrater, fetter och salter, som har viktiga funktioner. Mitt arbete har främst fokuserat på den viktiga roll som salivens proteiner utgör.

I saliven har man hittills hittat över 1000 olika proteiner och dessa har många varierande funktioner, varav en del överlappar varandra. Exempel på viktiga funktioner är antimikrobiell aktivitet som påverkar den mångfald av bakterier som också finns i saliven, smörjande egenskaper som underlättar tal och när vi tuggar, samt att motverka syra-angrepp från bakterier.

Protein består av små beståndsdelar som kopplas samman till långa kedjor. De små beståndsdelarna, s.k. aminosyror, kan ha olika strukturar och därmed olika egenskaper. Exempelvis kan de vara laddade/hydrofila (älska vatten) eller vara hydrofoba (dvs. sky vatten). Aminosyra-kedjans uppbyggnad; hur lång den är samt hur den veckar sig, har stor betydelse för proteinets funktion och egenskaper. De hydrofoba och hydrofila aminosyrorna i proteinet leder till att proteinet dras till gränsytor där båda sorter av aminosyror trivs, exempelvis mellan tandytan och saliven. Proteinet har då adsorberat; dvs. fäst vid, en yta.

I munnen finns många olika typer av ytor, exempelvis slemhinnan, tandytan samt även material som används för dentala konstruktioner. På dessa ytor fäster salivproteiner och den film som då bildas kallas pellikel. Pellikeln har många viktiga funktioner; bl.a. har den en inverkan på vilka bakterier som fäster till ytorna som senare bildar plack. Plack kan, om det inte tas bort, orsaka hål i tänderna eller tandlossning. Genom att förstå och kunna förutspå
pellikelns sammansättning och uppbyggnad samt hur komponenterna i filmen växelverkar med varandra vore det möjligt att utveckla nya och bättre metoder för plackkontroll.

I denna avhandling undersöks pellikel-bildningen i olika nivåer av komplexitet. Dels har inbindningen från enskilda salivproteiner undersökt för att förstå vilka grundläggande krafter som är viktiga vid filmbildningen. Därtill har system bestående av flera komponenter undersökt för att ge insikt i interaktioner mellan olika proteiner under filmens uppbyggnad. I studierna har ytans egenskaper, på vilken komponenterna binder, visat sig ha stor inverkan på filmen som bildas; dels med avseende på hur mycket protein som fäster på ytan men även på hur hårt proteinerna sitter fast. På liknande sätt som pellikeln byggs upp kan man bygga en film bestående av flera proteinlager (multilager). En sådan multilager-uppbyggnad påverkas av egenskaperna hos de samverkande komponenterna, samt av ytan. Hur de adsorberade filmerna påverkas av en tensidlösning (SDS; en typ av tvättmedelmolekyl som finns i tandkräm) har också undersökt. Man fann då att delar av de adsorberade proteinfilmerna lossnade från ytan. Vi kunde även se att genom att adsorbera flera komponenter efter varandra på samma yta, kan man minska fraktionen av enskilda proteiner som lossnar från ytan vid exponering för SDS. Detta kan bidra till att styra sammansättningen hos filmen till exempel öka relativ a andelen av speciella proteiner i filmen och därmed få en ökad effekt av deras funktioner.

För att kunna undersöka pellikelns sammansättning krävs att man kan få hela filmen att lossna från tandytan. För att möjliggöra detta har vi visat att genom att gnugga tandytan med en liten fiber-kudde indränk i 0.5 % SDS lösning kan man få hela pellikeln att lossna från tandytan. Dessutom har vi visat att sammansättningen på pelliklar från olika dentala material (emalj, titan-implantat och akryl-plast) skiljer sig åt, vilket därmed även kan leda till skillnader i sammansättningen av placket och därmed utvecklandet av orala sjukdomar.
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