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What interactions drive the salivary mucosal pellicle formation?

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ABSTRACT

The bound salivary pellicle is essential for protection of both the enamel and mucosa in the oral cavity. The enamel pellicle formation is well characterised, however the mucosal pellicle proteins have only recently been clarified and what drives their formation is still unclear. The aim of this study was to examine the salivary pellicle on particles with different surface properties (hydrophobic or hydrophilic with a positive or negative charge), to determine a suitable model to mimic the mucosal pellicle. A secondary aim was to use the model to test how transglutaminase may alter pellicle formation. Particles were incubated with resting whole mouth saliva, parotid saliva and submandibular/sublingual saliva. Following incubation and two PBS and water washes bound salivary proteins were eluted with two concentrations of SDS, which were later analysed using SDS-PAGE and Western blotting. Experiments were repeated with purified transglutaminase to determine how this epithelial-derived enzyme may alter the bound pellicle. Protein pellicles varied according to the starting salivary composition and the particle chemistry. Amylase, the single most abundant protein in saliva, did not bind to any particle indicating specific protein binding. Most proteins bound through hydrophobic interactions and a few according to their charges. The hydrophobic surface most closely matched the known salivary mucosal pellicle by containing mucins, cystatin and statherin but an absence of amylase and proline-rich proteins. This surface was further used to examine the effect of added transglutaminase. At the concentrations used only statherin showed any evidence of crosslinking with itself or another saliva protein.

In conclusion, the formation of the salivary mucosal pellicle is probably mediated, at least in part, by hydrophobic interactions to the epithelial cell surface.

1. Introduction

1.1. The bound mucosal pellicle

The oral mucosa has to be extremely tough to withstand the extreme conditions it is exposed to, such as the abrasive action and temperature extremes associated with an extremely wide range of foods in the human diet. This concerns both modern human diet as well as the pre-historic one; from hot beverages and fire-cooked meats, down to sub-zero frozen desserts, and tough grasses and vegetables (including various tubers) that contain highly abrasive silica particles (phytoliths) [1]. The oral cavity has two lines of defence; firstly, the parts of oral mucosa that are under direct action of mechanical forces such as the hard palate developed into mechanically tougher keratinised tissues, designed to protect the underlying cells from damage [2]. Secondly, the harsh mechanical environment of the oral cavity is tempered by the lubricating effect of the salivary pellicle that protects both tooth enamel and soft tissue [3–5], including softer non-keratinised oral surfaces such as for example buccal mucosa. The bound mucosal pellicle is a supra-molecular film with a complex architecture that comprises several structural layers. It comprises a complex of many salivary proteins including: sIgA, MUC5B, MUC7, carbonic anhydrase VI (CAVI) and cystatin S [6,7]. Salivary mucins, MUC5B and MUC7 are key for providing layer protection and lubrication due to their high molecular weight and high level of hydration which is due to the presence of highly glycosylated regions. Both type of salivary mucins are found to be strongly retained on the buccal cell

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surfaces [8,9], while within tooth enamel pellicle the mucin composition is dominated by MUC5B [6]. The self-assembly process of salivary proteins varies greatly depending on the type of oral surfaces, with variations in composition, protein content, thickness and the rate of replenishment. The key element of the assembly process is the formation of a tightly bound layer that ensures adhesion of the pellicle and also acts as a template for further protein/mucin assembly.

1.2. Formation of the bound mucosal pellicle

Adsorption of individual salivary proteins and whole saliva have been widely studied on different surfaces. Hydroxyapatite (HAP) has largely been studied as a model for the enamel pellicle [10]. Tooth enamel, being a mineral surface, has a number of distinct features. Thus, the enamel pellicle contains significant levels of statherin, proline-rich proteins, and CAVI, essential for remineralisation/demineralisation of the enamel [4,5]. Statherin has a particular affinity to the hydroxyapatite surfaces due to the presence of Ca^{2+} binding domains. By contrast, it has poor retention on the buccal cell surface [7], and hence is considered to be a specific constituent of the enamel pellicle [11]. Statherin, PRP-1 and PRP-3 have all shown the ability to bind to both hydrophobic and hydrophilic surfaces, but to a much lower extent on the later with exception of PRP-1, due to its lower negative net charge [12].

MUC5B contains both hydrophilic heavily glycosylated domains, and hydrophobic domains located within non-glycosylated areas [13]. MUC5B has also been shown to have stronger adsorption to hydrophobic surfaces, as opposed to hydrophilic, leading to higher adsorbed mass and slower desorption times [12,14]. The addition of calcium has also been shown to facilitate MUC5B deposition through promoting protein cross-links [15]. Unlike MUC5B, MUC7 has much smaller molecular weight (250 kDa versus over 2000 kDa for MUC5B) and comprises a single glycosylated region surrounded by relatively small non-glycosylated domains [16]. Due to a larger relative size of the glycosylated domains, MUC7 has much higher levels of hydration which effects weaker adsorption. However, MUC7 has high propensity to self-associate which can counteract its high solubility and increase incorporation into the pellicle due to physical entanglements and formation of complexes with lower molecular weight proteins such as IgA [17,18].

The process of salivary protein adsorption and binding onto surfaces is complex due to the number of proteins present, varying protein size and individual protein concentration. This complex process is governed by a finely tuned accord of electrostatic and hydrophobic forces, hydrogen bonds, as well as specific binding interactions and chemical cross-linking. Many factors can influence salivary film formation, for example, ionic composition can have a significant influence on pellicle development, through increased/decreased level of electrostatic interaction and protein cross-linking [15]. Despite shear multitude of interaction mechanisms, certain common interaction patterns did emerged. Thus, a number of research groups investigated the surface deposition/adsorption of saliva; it has been established that salivary proteins demonstrate much higher affinity to hydrophobic surfaces [14,19–22]. This goes in line with the fact that the bare oral mucosa is a largely hydrophobic surface, which becomes more hydrophilic as proteinaceous layer builds up [23]. Proteinaceous layers can be formed on hydrophobic surfaces from whole mouth saliva (WMS), parotid saliva (PS) and submandibular/sublingual saliva (SMSL). By contrast, on hydrophilic surfaces the deposited amounts are lower, which is particularly striking for PS that does not form a stable film on hydrophilic surfaces [24,25], which can be associated with the high concentration of salivary amylase in PS secretions. We note that most salivary proteins participate in pellicle formation. However there are notable exceptions, thus on oral epithelial cells amylase, one of the most abundant salivary proteins, shows minimal binding within the bound mucosal pellicle [7].

Alternative explanations suggested associate the degree of deposition with the presence of proteins such as transglutaminase (TGM) that can aid in protein cross-linking thereby facilitating pellicle formation [3,5,26]. Statherin and PRP-1 are among those shown to crosslink due the presence of TGM [27,28]. TGM3 has been confirmed to be present in the mucosal pellicle in both pro-enzyme form and in its active form [7]. However, the lack of statherin and PRPs in pellicles formed on various artificial substrates suggests that the role of TGM in the pellicle development is not always critical.

1.3. Aims

The aim of this study was to elucidate mechanisms of salivary binding by exploring which salivary proteins bind to hydrophobic, hydrophilic positive and hydrophilic negative charged particles using un-stimulated whole mouth saliva (UWMS), PS and SMSL. How strongly proteins bind and how well retained proteins are will be compared between saliva types. The role of TGM will also be investigated to see if this improves protein retention and aids in pellicle development. It is predicted that a set of particles with different surface chemistries will allow a more in-depth mechanistic insights that otherwise can be complicated by a complex nature of real biological surfaces. It will also mimic the chemically diverse spectrum of surfaces in the oral cavity and provide a suitable material to study mucosal pellicle development. Finally, if a suitable model is found, it could be used for further studies of the mucosal pellicle. This capability aspect of this work is of particular interest since enamel and soft tissue (e.g. buccal) mucosa surfaces require laborious sourcing, as well as raise considerable ethical considerations with studies in vivo.

2. Methods

2.1. Saliva collection

UWMS, PS and SMSL were collected from two volunteers, who refrained from eating, drinking and using mouth-cleaning products for 1 h prior to collection. UWMS was collected by drooling into universal tubes until 2 ml+ had been collected. PS was collected using a Lashley cup attached to one of the parotid glands and a citrus sweet was used to stimulate saliva production until 2 ml+. SMSL was also collected in a universal tube by blocking off the parotid glands with dental roll, which absorbs any secretion. A mucus-specimen trap was then used to draw up SMSL, which was allowed to pool in the bottom of the mouth following chewing stimulation. All saliva was collected fresh for each experiment and used immediately for incubation on the different particle types. UWMS was centrifuged before use at 5000 RPM for 5 min.

2.2. Particle preparation and saliva incubation

Different particles were selected for their different surface types: polystyrene (PSt) (hydrophobic) (Bangs Labs, Fisher, IN, USA), melamine formaldehyde (MF) (hydrophilic positive) (microParticles GmbH, Berlin, Germany) and silica (Si) (hydrophilic negative) (Kisker Biotech GmbH & Co. KG, Steinfurt, Germany). The particles were all stored in a liquid suspension and it was calculated that 100 μl, 200 μl and 400 μl of each suspension was need respectively to have approximately 405 cm^2 surface area, which would provide a surface area large enough for 1 ml of saliva to form a 7 nm thick film. All particle suspensions were topped up to 1 ml with PBS and water (1:1) (WPBS), which is a similar ionic concentration to saliva, and then centrifuged for 20 min at 10,000 rpm,