Journal of Dentistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Dentistry

journal homepage: www.elsevier.com/locate/jdent

Full length article

Oral astringent stimuli alter the enamel pellicle's ultrastructure as revealed by electron microscopy

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ARTICLE INFO

Keywords:
Salivary protein
Dental pellicle
Haemostatic agent
Astringency
SEM
TEM

ABSTRACT

Objectives: This electron microscopic study aimed at investigating effects of oral astringent stimuli on the enamel pellicle's morphology.
Methods: Pellicles were formed *in situ* within 30 min on bovine enamel slabs, fixed to individuals' upper jaw splints. The pellicle-coated specimens were immersed *in vitro* in seven diverse astringent solutions and subsequently analyzed by scanning electron microscopy (SEM), energy dispersive X-ray (EDX) spectroscopy, as well as transmission electron microscopy (TEM). Four biocompatible astringents, namely the polyphenol epigallocatechin gallate, the metal salt iron(III) sulfate, the basic protein lysozyme, and the aminopolysaccharide chitosan, were additionally applied *in situ*. After rinsing the oral cavity with these compounds, the pellicle's ultrastructure was imaged by SEM and TEM, respectively. Untreated pellicle samples served as controls.
Results: Exposure to polyphenols and lysozyme induced particularly thicker and electron-denser pellicles in comparison to the control pellicle with similar characteristics *in vitro* and *in situ*. In contrast, acidic chitosan and metal salt solutions, respectively, revealed minor pellicle alterations. The incorporation of Fe and Al into the pellicles treated with the corresponding inorganic salts was verified by EDX analysis.
Conclusions: Astringent-induced pellicle modifications were for the first time visualized by TEM. The ultrastructural alterations of the dental pellicle may partly explain the tooth-roughening effect caused by oral astringent stimuli.
Clinical significance: Astringents might modify the pellicle's protective properties against dental erosion, attrition, as well as bacterial adhesion, and by this means may influence tooth health. The findings may thus be particularly relevant for preventive dentistry.

1. Introduction

The dental pellicle is a 0.1–1 μm thick proteinaceous layer which forms instantaneously on the enamel's surface when in contact with saliva [1]. A two-step mechanism has been proposed for pellicle development: Small proteins rapidly adsorb to the tooth surface, followed by a slower, continuous adsorption of proteins to the already protein-coated enamel. As observed by transmission electron microscopy the initial pellicle layer is apparent as an electron-dense basal layer, whereas the outer layer consists of a less electron-dense film with globular and granular structures [2,3].

The protein film acts as a lubricant, protecting the teeth from attrition and abrasion, displays a barrier against erosive noxae, and has antibacterial properties. Besides these beneficial effects on tooth health, the pellicle also provides a basis for biofilm formation as several proteinaceous constituents facilitate bacterial adhesion [2].

Plant polyphenols such as epigallocatechin gallate found, e.g., in green tea extract [4], have been described to enhance the tenacity and thickness of the proteinaceous pellicle layer [5,6] and improve the pellicle's protective properties against erosive noxae [7,8]. As the ingestion of polyphenols is typically accompanied by a mouth-puckering sensation [9] and a loss of the pellicle's inherent lubricating properties associated with blunt teeth [10], a phenomenon also referred to as oral astringency [11], we asked whether astringent molecules generally modified the pellicle's morphology. Besides polyphenols, commonly-used haemostatic agents for oral interventions are perceived as oral astringents, such as the metal salts iron(III) sulfate and aluminum(III) chloride [12–15], alongside the aminopolysaccharide chitosan [16–18]. A fourth class of astringent substances comprises proteins with a high isoelectric point [16,19,20], for example, the antibacterial enzyme

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http://dx.doi.org/10.1016/j.jdent.2017.05.011
Received 26 March 2017; Received in revised form 16 May 2017; Accepted 17 May 2017

Table 1
Concentrations of the astringent test solutions and experimental conditions

Compound class	Test compound	Molecular weight [g/mol]	Concentration [mg/ml]	Experimental condition
Polyphenol	(−)-Epigallocatechin gallate (EGCG)	458	2.3	*in vitro/in situ*
	Tannic acid	~1700[#]	1.7	*in vitro*
	Red currant seed coat extract	> 5000	5.0	*in vitro*
Metal Salt	$Fe_2(SO_4)_3$	399	0.4	*in vitro/in situ*
	$AlCl_3{\cdot}6H_2O$	241	1.2	*in vitro*
Aminopolysaccharide	Chitosan	~500,000	1.0	*in vitro/in situ*
Basic Protein	Lysozyme	14,300	7.1	*in vitro/in situ*

[#]variable number of galloyl moieties per molecule.

lysozyme, which was found to be highly abundant in the acquired pellicle [21,22].

The present study tested the hypothesis whether oral astringency is generally linked to an alteration of the pellicle's morphology. Therefore, pellicles were formed *in situ* on bovine enamel slabs, exposed *in vitro* and *in situ* to the latter set of chemically diverse model astringents and analyzed on the ultrastructural level by electron microscopy (TEM and SEM).

Astringent-induced ultrastructural modifications of the dental pellicle may influence its protective properties in respect to dental erosion or attrition. Furthermore, results of this study may provide molecular insights into the mechanism of blunt feeling teeth as evoked by the administration of haemostatic agents.

2. Methods

2.1. Subjects

Two healthy volunteers (age: 29 and 30 years), both members of the clinical staff, entered the study. The participants fulfilled the inclusion criteria showing no evidence of active caries or periodontal disease. Their mean stimulated saliva flow rate exceeded 1 ml/min. Subjects refrained from eating and drinking anything besides water 3 h before taking part in the experiments. Ethical approval (238/03; 2012) was granted by the medical ethics committee of the Medical Association of Saarland, Germany. The volunteers received verbal and written information concerning the study and signed a consent form prior to their participation.

2.2. Enamel specimen preparation

Enamel slabs (3 × 4 × 1.5 mm) were prepared from the labial surfaces of bovine incisor teeth (BSE-negative, 2-year old cattle). After cutting cylindrical specimens from the teeth with a saw (Conrad Apparatebau Clausthal GmbH, Clausthal-Zellerfeld, Germany), they were progressively polished by wet grinding with up to 4000 grit (Buehler, Düsseldorf, Germany). The enamel samples were disinfected, following a sequential protocol: After ultrasonication in sodium hypochlorite (3%) for 3 min to remove the smear layer, the slabs were thoroughly rinsed with distilled water. The specimens were then washed for 5 min in ice-cold water and ultrasonicated in ethanol (70%) for 15 min. Finally, the specimens were stored for 24 h in distilled water.

2.3. Astringent stimuli

Test compounds used for *in situ* and *in vitro* experiments were applied at concentrations explicitly above their orosensory threshold for astringency [16]: (−)-Epigallocatechin gallate (EGCG) and $Fe_2(SO_4)_3$, respectively, were obtained from Sigma-Aldrich, St. Louis, MO, USA and dissolved in ultrapure H_2O at a concentration of 5 mM and 1 mM, respectively. Lysozyme (Sigma-Aldrich, Steinheim, Germany) was used at a concentration of 0.5 mM. Poly-(β-(1 → 4)-D-glucosamine (chitosan, degree of deacetylation > 92.6%, molecular weight ~500 kDa, Heppe Medical Chitosan GmbH, Halle/Saale, Germany) was dissolved in 0.1 M $HCl_{(aq)}$ and was further diluted with H_2O to gain a final concentration of 2 µM.

The following compounds were applied *in vitro* only: $AlCl_3{\cdot}6H_2O$ (Carl Roth, Karlsruhe, Germany) was used as a 5 mM solution to approximate the Al content in the commercial astringent mouth rinse Mallebrin®. Tannic acid (Caelo, Hilden, Germany) was used at a concentration in the order of 1 mM. To isolate the astringent, high molecular weight fraction from red currant seeds, 500 g fresh red currants (*Ribes rubrum*), which were bought on a local market, were squished and the seeds were mechanically separated from the fruit pulp, with the seed coats intact. The astringent seed coat liquid was extracted with 70% MeOH. The solvent was removed and the crude extract freeze-dried. The lyophilizate was taken up in H_2O and further fractionated by a Vivacell 250 static gas pressure ultrafiltration unit equipped with a 5 kDa molecular weight cutoff polyethersulfone membrane (Sartorius, Göttingen, Germany). After freeze-drying, the high molecular weight astringent retentate of the seed coat extract was used at a concentration of 5 mg/mL for *in vitro* experiments. Table 1 summarizes the concentrations of the astringent solutions and experimental conditions used within this study.

2.4. In situ pellicle formation

Individual splints were manufactured for the upper jaw from 1.5 mm thick methacrylate foils (Scheu-Dental, Iserlohn, Germany) using a thermoforming technique (Erkopress ES 2004, Erkodent GmbH, Pfalzgrafenweiler, Germany). The bovine enamel slabs were then mounted on the buccal side of the splints with a polyvinyl siloxane impression material (President Light Body, Coltène, Switzerland), exposing only the enamel surface to the oral environment.

For initial *in situ* pellicle formation the splints were carried in the oral cavity for 30 min. After removing the splints, the slabs were thoroughly rinsed with distilled water to remove loosely-attached saliva components.

2.5. In vitro experiments

Pellicle-coated specimens of both individuals (two samples each) were immersed *in vitro* for 1 min in one of the aqueous solutions of the astringents EGCG, tannic acid, red currant seed extract, $Fe_2(SO_4)_3$, $AlCl_3$, chitosan, and lysozyme, and then rinsed with distilled water. One of the slabs was prepared for scanning electron microscopy, the other slab for transmission electron microscopy.

2.6. In situ experiments

After initial *in situ* pellicle formation, two specimens were directly removed from the oral cavity to serve as control pellicles. Solutions (10 ml) of iron(III) sulfate, chitosan, EGCG, and lysozyme, respectively, were then used for intraoral rinsing (30 s). Two specimens were removed from the oral cavity immediately after rinsing with astringent, whereas a final set of two samples was left intraorally for 30 min. All