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Fluorescence microscopic visualization of non cellular components during initial bioadhesion *in situ*

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ARTICLE INFO

Article history:
Accepted 18 July 2013

Keywords:
Fluorescence microscopy
Oral cavity
Bioadhesion
Pellicle
Glucans

ABSTRACT

Objective: The formation of an intraoral biofilm is primarily determined by initial bioadhesion processes, including molecular interactions. Therefore, this study aimed to establish fluorescent labelling protocols to enable the simultaneous visualization of different pellicle enzymes, extracellular glucans and adherent bacteria throughout the initial phase of biofilm formation.
Design: *In situ* formed biofilm samples were collected on enamel and dentine slabs that were fixed on buccal sites of individual splints, being worn by 5 subjects. After an intraoral slab exposure from 30 min to 8 h, the following specially adapted fluorescent labelling assays were performed and analyzed by epifluorescent microscopy: pellicle-amylase, -lysozyme, -peroxidase and -glycosyltransferases B, C and D were marked with specific primary antibodies and then visualized by the aid of different fluorescently labelled secondary antibodies (Texas Red, DyLight 488, FITC). Afterwards the same samples were subjected to a combined DAPI-/Concanavalin A-staining to determine adherent bacteria and glucans.
Results: All fluorescence labelling assays were successfully established to visualize pellicle enzymes, glucans and adherent bacteria at different times of biofilm formation. The combination of the labelling protocols showed a characteristic agglomeration of glucans and bacteria as well as an increased concentration of the pellicle enzymes in the initial phase of bioadhesion.
Conclusion: Fluorescent labelling techniques are a valuable supplement of dental research as they provide an insight into the mutual interactions of different biofilm determinants *in situ*. Based hereon, information could also be deduced about the influence of oral therapeutics on individual caries susceptibility.

1. Introduction

The complex phenomenon of biofilm formation in the oral cavity is a central field of interest in dental research.[1–3] This particularly involves the establishment of reliable methods to precisely uncover the physiological molecular and cellular interactions promoting bioadhesion *in vivo*. Several research approaches have gradually gathered a wide range of information about the components as well as the formation process of

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0003–9969/$ – see front matter
http://dx.doi.org/10.1016/j.archoralbio.2013.07.006

the initial biofilm.[4–7] The tooth is covered by the acquired pellicle, which, due to biomolecule adsorption and remodelling, mediates the interactions between the tooth surface and the surrounding moist oral environment. It contains important structural components which either expose protective antibacterial properties, such as lysozyme and peroxidase or, considering the remarkable proportion of amylase and bacterial glycosyltransferases (GTF), provide essential preconditions for a bacterial colonization. Especially in presence of starch hydrolysates pellicle bound GTF B and C form predominantly insoluble glucans, differing in the type of α-glucosidic-linkages whereas GTF D synthezises soluble glucans and acts as a primer fascilitating the binding of other isoforms, respectively.[8,9] These matrix components form a scaffold for the development of a bacterial biofilm as they provide a nutrient reservoir, expose bacterial binding sites and enhance the mechanical stability by enforcing the aggregation of microorganisms.[10–12]

Considering the structural complexity of the initial biofilm, important molecular interactions in between the structural components, with the tooth-surface as well as with microorganisms are assumed and were partially corroborated by some studies. Thus it was shown *in vitro* that pellicle bound GTF B and C form more 3-linked glucans in presence of starch hydrolysates than GTF B and C in solution.[13] Furthermore it can be assumed that the simultaneous glucan formation of pellicle bound GTF B and C facilitates microbial attachment, aggregation and formation of microcolonies.[14,15]

The current state of research discloses reasonable insights into the concentration and the function or enzymatic activity of pellicle proteins.[4,5,7] Especially the gold-immunolabeling technique in combination with SEM or TEM imaging is, due to its particularly high resolution, a valuable method to visualize the ultrastructure of the pellicle as well as to detect specific antigen providing molecules.[16] Information can be obtained about the presence, the quantity and the distribution of certain single pellicle components. Although the value of this method is unquestionable regarding the analysis of specific pellicle components, it is only applicable for one type of labelled molecules at a time and the preparation of the samples is rather complex. Moreover, efforts were also made to determine different pellicle proteins by mass spectrometry.[17] Additionally, enzyme activity measurements expose further qualitative information about specific functional properties of pellicle samples.

In contrast, less is known about the dynamics of molecular interactions. The mentioned techniques all fail to enable a simultaneous visualization of different biofilm components, especially non-cellular constituents. And yet this is important for the better understanding of general molecular interactions, the dynamic exposure of pellicle components whilst biofilm-maturation as well as interaction-mediated increase or reduction of pellicle components.[18] A few recent study approaches describe the simultaneous visualization of glucans and microorganisms by fluorescent labelling and confocal microscopy.[9,14,19] They indicate the dynamics of glucan formation as being related to bacterial presence, however, the experiments were all conducted *in vitro*. Fluorescent labelling is, if based on appropriate protocols and specific markers, an attractive and simple method to simultaneously visualize different cellular and extracellular structures without disturbing their natural interference. Therefore, it was the aim of this study to develop fluorescent labelling techniques on the basis of established research methods and under consideration of the special requirements evolved by the adherence of the *in situ* gained oral biofilm. Finally, conclusions might be drawn about the interaction of different enzymes described above with a special focus on their impact on glucan formation.

2. Methods

2.1. *Subjects*

The group of volunteers was formed of 5 healthy members of the laboratory staff who presented a good general and oral health with no evidence of gingivitis or periodontitis.

Beforehand, the legitimacy of the study was ensured by the ethic committee approval (EK 275092012) and all subjects have given their informed written consent. As described previously, *in situ* pellicle formation was performed on round enamel and dentine slabs (5 mm diameter) gained from the labial surfaces of bovine incisors of two-year-old cattle (BSE-negative).[20,21] First, the surface was wet-grinded by up to 4000 grit abrasive paper and if exposure of the dentine surface was intended, the enamel was furthermore removed.

Afterwards sequential disinfection protocols were carried out with the aid of ultrasonication.[21,22] The enamel slabs were kept in 3% NaOCl for 3 min followed by another 10 min in 70% ethanol. In between they were washed twice in destilled water and were finally stored in it for 24 h to enforce hydratization of the enamel surface. Differently, disinfection of the dentine surface was achieved by 1 min exposure to 70% ethanol, air drying and additional smear-layer removel by 1 min in 3% EDTA. And again, the slabs were washed twice and then stored in destilled water. To enable the intraoral application the slabs were fixed in buccal cavities located in region of the upper premolars and the first molar of individual upper jaw splints with a polysiloxane impression material (Provil® novo light regular, Haraeus Kulzer, Germany). Up to 8 slabs were attached to each splint and were carried intra-orally for up to 8 h without intake of food. After 30 min, 2, 4, 6 or 8 h the slabs were removed from the splints and rinsed for 15 s with 0.9% NaCl solution to eliminate non-adsorbed salivary remnants.

Eventually various fluorescent and immunofluorescent labelling protocols were performed to detect pellicle amylase, peroxidase, lysozyme, GTF B, C, D, glucans as well as bacteria and visualize them simultaneously in the same samples.[21,23] Due to the varied intraoral exposure times of the slabs information were gained about the presence and relation of the corresponding molecules throughout different stages of biofilm formation.

2.2. *Determination of pellicle enzymes by immunofluorencence*

The core of this study was the implementation of a two-step immunofluorescent labelling assay which was adapted on the