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In situ demineralisation of human enamel studied by synchrotron-based X-ray microtomography – A descriptive pilot-study

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ABSTRACT

An *in situ* study was designed to investigate naturally developed demineralisation in human enamel in a widely non-destructive manner in combination with X-ray microtomography. Samples of human enamel were carried in the oral cavity of participants for 24 h daily for either 21 or 29 days using so-called intraoral mandibular appliances (ICTs). Demineralisation was thereby generated in a natural way without causing caries in the subjects' dentition. By employing synchrotron-based X-ray microtomography (XMT) in combination with volume image analysis, a quantification and three-dimensional visualisation of different stages of mineral density loss was possible. Basic features of the demineralised samples were similar to those reported in earlier *in vitro* studies. However, the analysed samples showed significant differences in the morphology of surface attack and the degree of mineral density loss depending on the carrier, the exposure time and the position within the ICT. In particular, the varying local conditions within a carrier's oral cavity seem to be different than in an *in vitro* study. Our results show that the combination of ICTs and quantitative image analysis applied to XMT data provides an analytical tool which is highly suited for the fundamental investigation of naturally developed demineralisation processes.

1. Introduction

Several studies attempting to understand the kinetics of early demineralisation which starts underneath the enamel surface have been performed in the past. Imaging techniques such as optical microscopy (Darling, 1958) and electron microscopy (Haikel et al., 1983; Hicks and Silverstone, 1985; Jones and Boyde, 1987; Pearce and Nelson, 1989; Shellis and Hallsworth, 1987; Boyde et al., 1995, 1999) were frequently used. Those techniques have the disadvantage of penetrating the material under study a few nanometres to 1 μm only. Therefore, the resulting two-dimensional images provided a limited insight into the lesion development and required invasive tissue preparation for the investigation of structural changes below the surface of the specimens (Baumgartner et al., 2000).

An alternative approach is the application of X-ray micro computed tomographic (XMT) techniques which provide three-dimensional images of the scanned specimen (Hsieh, 2009; Stock, 2008; Buzug, 2008). Numerous articles reporting on enamel demineralisation using high resolution computed tomography have been published which (*e.g.*, Bing et al., 2011; Hamba et al., 2011; Schwass et al., 2009; Tanaka et al., 2010). Frequently synchrotron radiation is employed for XMT in order to achieve higher spatial resolution and contrast (Bonse and Busch, 1996). At a synchrotron light source the available photon flux density (Photons/mm^2/s) is orders of magnitude higher and the beam propagation is quasi parallel. The latter allows for extending the distance between source and sample up to several 100 m. Consequently, unlike in conventional radiography which is based on X-ray tubes, the influence of the finite X-ray source on the image resolution is surpressed (*cf.*, *e.g.*, Rack et al., 2008b). Furthermore, the high flux allows the use of monochromators. Monochromatic radiation increases the

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contrast while reducing beam-hardening artefacts (Brooks and Di Chiro, 1976).

Today, X-ray microtomography (XMT) is a mature technique with high potential for dental research (Delbem et al., 2009). Working in absorption contrast, the images acquired with (quasi-) monochromatic radiation allow for a quantitative measure of changes in the degree of demineralisation inside, *e.g.*, enamel tissue. Several studies attempting to understand the three dimensional kinetics of demineralisation have been performed using XMT (Kernen et al., 2008; Kinney et al., 1994, 1995; Anderson and Elliott, 2000; Stock, 2008; Farah et al., 2009; Huang et al., 2007; Delbem et al., 2009; Kielbassa et al., 2000; Dowker et al., 2003, 2004; Davis and Wong, 1996; Elliott et al., 1981), even though extrapolating the three-dimensional de- and remineralisation processes from such a measurement is a non-trivial task (Dowker et al., 2003).

Some studies found in the literature are based on *in vitro* investigations, *i.e.* the demineralisation is artificially produced outside the oral cavity. Obviously, the clinical relevance of *in vivo* studies is significantly higher, in particular due to the multi-factorial nature of dental caries (ten Cate, 1996; Zero, 1995). Unfortunately, XMT is inapplicable for human dental *in vivo* studies for multiple reasons. One reason is the high mutagen potential of the employed hard X-ray radiation. Hence, demineralised teeth have to be extracted prior to imaging (Dowker et al., 2004; Huang et al., 2007, 2010). This makes time-resolved investigations of demineralisation processes impossible. Furthermore, information on the duration of and the conditions during demineralisation is limited.

As an alternative, we propose an *in situ* study using intraoral appliances (intra-oral cariogenity test systems, ICT) which allow for an exposure of enamel samples to the human oral cavity over longer periods of time. Consequently, they represent promising tools to study naturally developed demineralisation in human oral cavities without causing any decay of the natural dentition (Koulourides and Volker, 1964; Koulourides and Chien, 1992). Thus, *in situ* studies combining ICTs with XMT imaging of the demineralised samples may bridge the gap between the highly controlled laboratory condition *in vitro* and the uncontrolled clinical situation *in vivo*. It is the aim of our pilot study to test the applicability of these techniques for the investigation of natural demineralisation processes. To the best of our knowledge such studies have not been performed so far. Of particular interest is whether the enamel demineralisation differs in its morphology from artificially generated lesions.

Furthermore, earlier studies focused mainly on the evaluation the three-dimensional (3D) image data by means of volume renderings and density profiles along line sections. In our work methods from quantitative volume image analysis are introduced. Their application allows one to fully exploit the information provided by the 3D image data.

2. Materials and methods

2.1. Preparation of specimens and intraoral appliance

Eight caries free third molars were extracted and stored for 24 h in Ringer's solution (DAB 7; Delta-Pharma, Pfullingen, Germany). After extraction the remaining tissue was removed with a scalpel under running water. With a diamond-coated wire saw (wire: 300 μm; saw: Type 4240, Well, Mannheim, Germany) the crowns were separated from the roots. During the sawing process the teeth were wetted constantly with phosphate enriched Na-Cl solution. 33 cubes of roughly 1 mm^3 volume consisting of enamel of the occlusal surfaces and parts of contiguous dentine were prepared.

The specimens were covered with a varnish coat on five faces, leaving only the natural outer (occlusal) enamel surface free (Nail varnish: Nivea, Beiersdorf, Hamburg, Germany). The cubes were sterilised for 25 min at 1.8 bar and 122 °C (Technoclav 50, Technomara, Fernwald, Germany).

Afterwards 32 samples were randomly integrated into four removable ICTs, as shown in Fig. 1. The assembled samples were covered with a gold-micro mesh that allowed for accumulation of plaque on the specimen surface.

2.2. Participants and experimental setup

Four participants volunteered for the pilot-study (3 female, 1 male, age: 23–27 years). The participants signed appropriate information consent forms. They were in good general health, had no infectious diseases and did neither take antibiotics nor medications that affect salivary flow for at least twelve months before and during the study. All participants were non-smokers and had moderate past caries experience with restored decays. If necessary, radiographs were taken to exclude caries and all carious lesions were restored by a dentist of the Department of Conservative and Preventive Dentistry, RWTH Aachen University, Germany. The participants had a minimum of 28 natural teeth and generally good periodontal health. They wore the ICT day and night and removed it only for individual oral hygiene with fluoride toothpaste and to clean the ICT twice a day with fluoride-free toothpaste. To allow plaque to grow under the micro mesh the participants were instructed to clean the area of the specimens only by using running water. We chose different time periods of carrying the samples, presuming demineralisation to emerge after a period of at least three and at most four weeks. Since the time period for the XMT measurement was constricted to several hours during one day, all samples had to be removed from the ICTs on the same day. Therefore, two research subjects (participants A and B) started with the *in situ* study 21 days and two subjects (participants C and D) 29 days before the day of measuring. After extraction and during XMT measurement each sample was stored in a labelled closed container filled with Ringer's solution. A total of seven samples from participant A, eight from participant B, seven from participant C and one from participant D were scanned by XMT.

2.3. Synchrotron-based microtomography using hard X-rays

The XMT measurements were carried out at the BAM*line*, the first hard X-ray beamline of the BESSY–II light source (Helmholtz Zentrum für Materialien und Energie, Berlin, Germany, *cf.* Rack et al., 2008b). Quasi-monochromatic radiation of 25 keV X-ray photon energy was attained by setting a double-multilayer monochromator (approximately 1% energy bandwidth). Attenuators were inserted to block photons passing the monochromator by means of total reflection. To convert the synchrotron light after transmitting the sample into visible light, a 7-μm-thick YAG:Ce (Ce-doped $Y_3Al_5O_{12}$) single-crystal scintillator was used. The scintillator was coupled *via* diffraction-limited visible light optics to a CCD camera (VersArray: 2048B, 2048 × 2048 pixel, Princeton Instruments). The indirect detector samples the visible light image with 1.6 μm effective pixel size. The spatial resolution achieved is approximately 7–8 μm. The images were taken in absorption mode, *i.e.* the detector was placed close downstream of the samples, which allows for neglecting coherence effects on our results, *i.e.* X-ray inline phase contrast (Zabler et al., 2007). Each specimen was scanned with 900 projections over a 180° rotation. Three-dimensional data sets were reconstructed using the filtered backprojection algorithm (Kak and Slaney, 1988) implemented in