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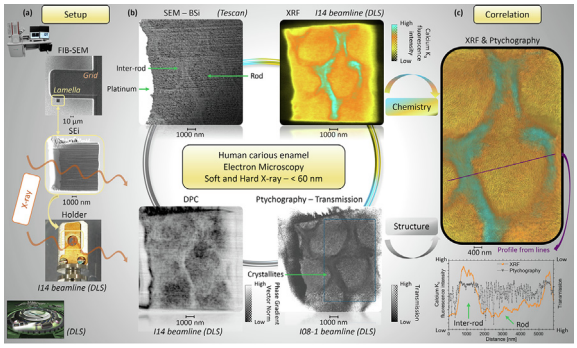
Cyril Besnard [a,*], Ali Marie [a], Sisini Sasidharan [a], Petr Buček [b], Jessica M. Walker [c], Julia E. Parker [c], Thomas E.J. Moxham [a,c], Benedikt Daurer [c], Burkhard Kaulich [c], Majid Kazemian [c], Richard M. Shelton [d], Gabriel Landini [d], Alexander M. Korsunsky [a,*]

[a] MBLEM, Department of Engineering Science, University of Oxford, Parks Road, Oxford, Oxfordshire OX1 3PJ, UK
[b] TESCAN-UK Ltd., Wellbrook Court, Girton, Cambridge CB3 0NA, UK
[c] Diamond Light Source Ltd., Didcot, Oxfordshire OX11 0DE, UK
[d] School of Dentistry, University of Birmingham, 5 Mill Pool Way, Edgbaston, Birmingham, West Midlands B5 7EG, UK

GRAPHICAL ABSTRACT



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ABSTRACT

This study reports the characterisation of human dental enamel caries using synchrotron nanoscale correlative ptychography and spectroscopic mapping in combination with scanning electron microscopy. A lamella ~2.4 µm thick was extracted from a carious enamel region of a tooth using focused ion beam-scanning electron microscopy and transferred to two synchrotron beamlines to perform hard X-ray nano-fluorescence spectroscopy simultaneously with differential phase contrast mapping at a beam size of 55 × 45 nm. Soft X-ray ptychography data was then reconstructed with a pixel size of 8 nm. The two dimensional variation in chemistry and structure of carious enamel was revealed at the nanoscale, namely, the organisation of hydroxyapatite nano-crystals within enamel rods was imaged together with the inter-rod region. Correlative use of electron and X-ray scanning microscopies for the same sample allowed visualisation of the connection between structure and composition as presented in a compound

* Corresponding authors.
E-mail addresses: cyril.besnard@eng.ox.ac.uk (C. Besnard), ali.marie@eng.ox.ac.uk (A. Marie), sisini.sasidharan@eng.ox.ac.uk (S. Sasidharan), petr.bucek@tescan.com (P. Buček), jessica.walker@diamond.ac.uk (J.M. Walker), julia.parker@diamond.ac.uk (J.E. Parker), thomas.moxham@eng.ox.ac.uk (T.E.J. Moxham), benedikt.daurer@diamond.ac.uk (B. Daurer), burkhard.kaulich@diamond.ac.uk (B. Kaulich), majid.kazemian@diamond.ac.uk (M. Kazemian), R.M.Shelton@bham.ac.uk (R.M. Shelton), G.Landini@bham.ac.uk (G. Landini), alexander.korsunsky@eng.ox.ac.uk (A.M. Korsunsky).

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X-ray ptychography
FIB-SEM

image where colour indicates the relative calcium concentration in the sample, as indicated by the calcium K_α fluorescence intensity and grey scale shows the nanostructure. This highlights the importance of advanced correlative imaging to investigate the complex structure-composition relationships in nanomaterials of natural or artificial origin.

1. Introduction

Caries is a global disease with a prevalence in permanent teeth estimated to have affected 2.3 billion people in 2017 [1]. Lack of significant progress over many decades in combatting this disease suggests that the caries challenge could be attempted by combining methods and approaches from different research fields using complementary state-of-the art characterisation techniques. The focus of the present study was the combined nanoscale characterisation of dental carious enamel structure and composition which has not been done before. These technical advances which help in the analysis of the caries to reveal details in 2D at the nanoscale may also be applicable for characterisation of a variety of other materials.

Enamel forms the hard outer layer of the tooth crowns and has a fascinating hierarchical structure with micron scale rods formed by bundles of nano-crystallites of hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) [2]. This complex structure provides outstanding mechanical properties in terms of the combination of hardness and fracture toughness [3]. However, under acidic conditions resulting from the modern diets typically rich in fermentable sugars in combination with the metabolism of certain bacterial species organised into dental plaque, the enamel structure demineralises [1]. This leads to the loss of material and an increase in porosity in certain locations that undermines its mechanical properties, leading to the formation of caries [1]. Disrupting the caries process has been the objective of numerous research studies; however, decisive progress remains elusive [1,2]. One of the challenges is understanding and characterising the fine scale and complex organisation of HAp nano-crystallites. Such approaches require the combination of high-resolution imaging in both two and three dimensions (2D and 3D) to reveal structure, composition, crystallinity and orientation of the crystallites. The structure and chemistry of enamel needs to be analysed jointly with correlative studies and at different scales because of the complexity of the tissue. Recent studies have addressed the source of demineralisation at the nanoscale using for example, transmission electron microscopy (TEM) and atom probe tomography [4,5]. However, to understand caries fully there is also the need to analyse larger regions of enamel using state-of-the-art techniques that can potentially be applied in 3D.

Ptychography is a lens-less technique based on the reconstruction of overlapping coherent diffraction patterns. It can generate high resolution images [6] and can additionally be used in a tomographic mode for 3D imaging. It can also be combined with X-ray fluorescence spectroscopy (XRF) to map sample chemistry [7]. A related and also relevant technique that can be readily implemented simultaneously is differential phase contrast (DPC) imaging, based on extracting the phase information from the angular deflection of the beam [8].

The results report the successful use of synchrotron hard and soft X-ray nanoprobes for 2D mapping of chemical-structural detail in carious enamel down to 50 and 8 nm pixel size. This provides opportunities for the analysis of carious tissues samples as well as mimetic materials and remineralised layers to qualify and quantify the modifications and potential restoration of the enamel nanostructure. Importantly, this demonstrates the capabilities of X-ray nanoprobe techniques for the assessment of representative 'thick' samples of ~2.4 μm compared with TEM lamellae.

The micron-thick lamella sample, ~2.4 μm, was extracted from a block of carious enamel using a focused ion beam (FIB) lift-out method as previously described [1,2] from a carious location (identified as 'Location 12' in a previous study [2]). FIB-scanning electron microscopy (SEM) (Tescan Lyra 3, Tescan, Czech Republic) was used to attach the sample to a nanomanipulator needle and lifted out to a copper grid using milling steps and platinum deposition as described elsewhere [2], but without any further polishing once the sample was attached to the grid. The lamella thickness was determined using SEM. Secondary electron and backscattered electron images (SEi and BSi) were acquired using an accelerating voltage of 5 keV to visualise the sample before and after attachment (Figure: a,b). Two beamlines namely, I14 and I08-1 at Diamond Light Source (DLS, U.K.) were used for synchrotron X-ray analysis. The I14 nanoprobe instrument used X-rays with an energy of 8 keV focused to a beam size of 55 × 45 nm. XRF elemental mapping (backscatter geometry) was used to plot calcium (Ca) (K_α emission) integrated intensity simultaneously with the use of DPC (transmission geometry) using raster imaging with steps of 50 nm (in the x and y directions). The DPC phase gradient vector norm was calculated from the centre of masses of the deflected beam extracted using a bespoke I14 (DLS) script. The I08-1 soft X-ray instrument was used for ptychographic imaging using an energy of 1 keV in a vacuum (transmission geometry). Based on the acquired diffraction patterns, ptychographic reconstructions were performed using the PtyPy framework [9] using 100 iterations of the difference map algorithm followed by 100 iterations of the maximum likelihood algorithm. This provided a transmission map of the object at a pixel size of 8 nm and a spatial resolution estimated to be in the order of 20–30 nm. The analysis of the XRF map and ptychography images were performed using DAWN Data Analysis WorkbeNch software (DLS, U.K.), Matlab (MathWorks, USA) and Avizo software (Thermo Fisher Scientific, USA), along with the analysis of profile from lines with a step of 8 nm plotted with OriginPro (OriginLab Corporation, Northampton, MA, USA).

SEM imaging of the lamella prior to lift out was used to identify the rod and inter-rod regions and provided clear evidence of porosity due to preferential demineralisation in specific regions in the sample. Demineralisation was prevalent in the rods, whilst the inter-rod and boundary regions, where the sheath region has been described [1,2], appeared to be more resistant to acid attack (Figure: b). FIB-SEM imaging also suggested that enamel demineralisation led to structural alterations in comparison with non-carious regions [2]. It is important to note that lifting out the lamella led to a reduction in the clarity of the enamel structure that was likely to be due to ion redeposition and platinum deposit during the lift-out procedure (Figure: a).

The I14 nanoprobe combined the analyses of chemistry and structure at a pixel size of 50 nm and confirmed the non-uniform distribution of Ca in the sample (Figure: b); higher intensities of the Ca K_α fluorescence line in the measured XRF spectra were found in the inter-rod regions in comparison with the rods. Increased demineralisation in the rods was also observed using

DPC mapping through contrast variation (Figure: b). The carious enamel structure was revealed with greater clarity than seen using SEM due to the improved signal-to-noise ratio from the larger interaction volume (thickness). Although the nominal resolution was less than using SEM, these techniques showed the potential for improved contrast and for being applied to 3D imaging. The combined imaging, mapping modalities and contrast formation mechanisms offer complementary insights compared with electron microscopy techniques.

The resolution achieved in these experiments did not allow clear visualisation of the individual crystallites. In pursuit of further improvement, ptychographic imaging using soft X-rays was carried in the vacuum and the data was reconstructed with a pixel size of 8 nm. A careful alignment of the images obtained using soft and hard X-ray nanoprobes as well as SEM enabled linking of the images thus acquired. The transmission map obtained from ptychography revealed the fine detail of the crystallite organisation (Figure: b) which was evident within the rods and less prominent in the inter-rod regions: within a given rod the crystallites followed a "flow pattern" possibly associated with the mode of calcium phosphate deposition and crystallisation by ameloblast activity [1], while inter-rod regions that form a "filler" substance that ensures the continuity of enamel material, appeared to possess a less clear, more 'amorphous' structure. This provided an additional indirect confirmation that highly oriented and crystalline rod matter. It was notable that in the lamella from the carious region, the rod material showed greater susceptibility to acid demineralisation (chemical dissolution) compared with the boundary inter-rod substance.

The combination of structural details and elemental analysis at the nanoscale delivered rich information about carious enamel. This is illustrated in Figure: c which represents the superimposition and correlation of a XRF Ca K_α fluorescence intensity map with the ptychography transmission map from the same region of interest. The intensities of the Ca K_α fluorescence line in the measured XRF spectra of the lamella was appreciably higher in the inter-rod composed of different arrangements of crystalline materials than the highly oriented rods. From the profile from lines of the two modalities, additional visualisation of the correlation was highlighted showing the evolution of the Ca K_α fluorescence intensity from inter-rod to rod. A direct correlation with the increase in Ca K_α fluorescence intensity in inter-rod matching with the ptychography data and less variation in the transmission was observed. This correlation found at the nanoscale highlights the importance of this study at such scale ranges. This is a major step in the understanding of the physico-chemical nanoscale mechanism of caries demineralisation in preferential directions that requires further exploration and detailed mapping, including in 3D.

Previous studies of human dental tissue chemistry and structure using XRF and ptycho-tomography separately revealed the organisation of cementum and dentine using a beam size of 250 nm [7], and of dentine with a pixel size of 65 nm [6], respectively, but no significant studies on enamel. In the present study, higher resolutions were achieved and the benefit of combining information from the two techniques on the same sample were shown. To the best of the authors' knowledge, this level of detail has not been reported using these techniques. The findings have major implications not only for dental research and enamel repair [3], but also for other fields of study, e.g., bone repair. The techniques reported have advantages compared with nanoscale characterization techniques such as TEM [4] and polarisation-dependent imaging contrast mapping previously used for enamel [10], as those techniques require examination of much smaller thicknesses or only deliver surface analysis. In addition, the possibility to use a thicker sample in comparison with the above mentioned methods is beneficial due to the reduction of the damages and artefacts by FIB during the preparation of the sample [1,2] as a large thickness is probed.

In conclusion, the analysis of a thick lamella from carious dental enamel was successfully characterised at a pixel size of 50 nm using XRF and DPC for chemical and structural analysis. This was complemented with ptychography data reconstructed with a pixel size of 8 nm to reveal the details of crystallite arrangement. Preferential demineralisation revealed by Ca K_α fluorescence intensity inhomogeneity was linked to the difference in crystallite orientation between rod and inter-rod regions. The superimposition of imaging modes highlights the benefit of correlative analysis using different modalities.

Data availability statement

Data collected and interpreted in this study is maintained by the authors and can be made available upon request.

CRediT authorship contribution statement

Alexander M. Korsunsky and Cyril Besnard Conceptualization, Supervision with Gabriel Landini and Richard M. Shelton. Cyril Besnard, Ali Marie, Sisini Sasidharan, Jessica M. Walker, Julia E. Parker and Alexander M. Korsunsky Methodology, Data curation in the I14 synchrotron beamtime. Cyril Besnard, Ali Marie, Sisini Sasidharan, Thomas E.J. Moxham, Benedikt Daurer, Burkhard Kaulich, Majid Kazemian and Alexander M. Korsunsky Methodology, Data curation in the I08-1 synchrotron beamtime. Cyril Besnard Investigation, Methodology, Data curation, performed FIB-SEM analysis, sample preparation with support of Petr Buček. Cyril Besnard Investigation, Methodology, Formal analysis, Software, Visualization, analysed the data from the synchrotron and initially created the figure. Cyril Besnard Writing - Original Draft and Cyril Besnard, Petr Buček, Ali Marie, Sisini Sasidharan, Jessica M. Walker, Julia E. Parker, Thomas E.J. Moxham, Benedikt Daurer, Burkhard Kaulich, Majid Kazemian, Richard M. Shelton, Gabriel Landini, and Alexander M. Korsunsky Writing - Review & Editing.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Alexander M. Korsunsly Editor-in-Chief of JMADE.

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U.K.). Thanks to Diamond Light Source for the image of the synchrotron used in the Figure, https://www.diamond.ac.uk/PressOffice/MediaResources.html with acknowledgment of the copyright ©Diamond Light Source, 2022.

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