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A novel electron-microscopic method for measurement of mineral content in enamel lesions

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ABSTRACT

Objective: To assess Scanning Electron Microscopy in Back-Scattered Emission mode (BSE-SEM) for measurement of lesion mineral content as a function of depth. Direct comparison is made with Transverse Micro-Radiography (TMR) and Surface Micro-Hardness (SMH) on carious and erosive lesions.

Design: Caries lesions prepared from sound bovine enamel at 37 °C and pH 4.6 in unsaturated (7d) or part-saturated (8d, 4.1 mM Ca^{2+}, 8 mM Pi) lactic acid /methyl cellulose gel system, followed by TMR analysis. Erosive lesions prepared from sound bovine enamel (1% citric acid, pH3.8, room temperature) for 5, 10, 15 or 20 min at n = 10 per treatment group. SMH readings (Vickers diamond, 1.9 N, 20 s) were taken from acid-treated and reference areas of each sample. BSE-SEM performed on polished cross-sections of lesioned samples (Jeol JSM6490LV SEM; high vacuum, 10 keV beam voltage, magnification x500 with constant working distance of 10 mm). Under identical SEM conditions, polished standards i.e. MgF_2, alumina, Mg, Al and Si provided a calibration plot of BSE-SEM signal vs. atomic number ($\bar{z}$). Mineral content vs. depth plots were derived from the cross-sectional BSE-SEM data.

Results: Cross-sectional BSE-SEM images clearly differentiate between caries and erosive lesions. Comparison of caries lesion mineral loss from BSE-SEM with TMR data showed good correlation (R^2 = 0.98). Similarly, comparison of BSE-SEM data from erosive lesions showed good correlation (R^2 = 0.99) with hardness loss data from SMH.

Conclusion: BSE-SEM provides a relatively rapid and cost-effective method for the assessment of mineral content in demineralised tooth enamel and is applicable to both caries and erosive lesions.

1. Introduction

Caries lesions tend to form in localised areas of the teeth when food containing sugars or starch is converted to acids by bacteria which are present on the tooth surface, particularly in and around dental plaque. The consequent dissolution and removal of the enamel produces lesions which may extend beyond a depth range of 100 µm and ultimately result in cavity formation. In contrast, erosive lesions are caused by intrinsic (stomach acids) or extrinsic acids (from food or drink) with resultant softening and removal of enamel across the whole tooth surface. The effect of this acid erosion extends from, typically, ~0.1 µm depth in the very early stages to beyond ~100 µm after prolonged exposure. Despite the substantial reductions in caries effected by water fluoridation, topical fluoride application and particularly fluoride toothpastes, it remains ubiquitous worldwide. There is, in fact, evidence that the trend may be in reverse (Bagramian, Garcia-Godoy, & Volpe, 2009). In addition, acid erosion has recently been described as “an increasingly relevant problem” (Milosevic, 2017).

Hence there is a continuing need for accurate quantification of the mineral content of dental hard tissues, during in vitro and in situ demineralisation and remineralisation studies, in order to aid the design of clinical strategies to combat caries and erosion. A range of analytical techniques have been used for the assessment of mineral content in enamel lesions and reviewed extensively (Attin & Wegehaupt, 2014; Grenby, 1996; Schlueter, Hara, Shellis, & Ganss, 2011). These techniques include standard Radiography (Heaven, Firestone, & Feagin, 1990), Transverse Micro-Radiography (TMR) (Amaechi, Higham, & Edgar, 1998; Hamba, Nikaido, Nakashima, Sadr, & Tagami, 2011),

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Secondary Ion Mass Spectrometry [SIMS] (Frostell, Larsson, Lodding, Odelius, & Petersson, 1977), Electron Probe Micro-Analysis [EPMA] (Ngo et al., 1997), Surface Micro-Hardness (SMH) and Cross-Sectional Micro-Hardness (CSMH). For erosion studies, SMH tends to be the technique of choice with profilometry-based techniques also providing further physical information on topography and erosion crater depth (Elton, Cooper, Higham, & Pender, 2009; Gracia, Brown, Rees, & Fowler, 2010). SMH is relatively rapid but provides no structural information whereas CSMH is limited markedly by the number of indents which can be taken across shallow erosive lesions. While Transverse Micro-Radiography is considered to be the "gold standard" for quantification of mineral content, a very small number of research groups globally have used the technique for erosion studies compared to a significantly higher number for caries studies. This is mainly related to the relatively poor ability of Transverse Micro-Radiography to accurately profile mineral loss within the upper 5–10 μm of the enamel surface (Amaechi et al., 1998; Hall et al., 1997). This inevitably limits its application to the measurement of mineral content in erosive lesions which tend to have relatively shallow depths in the range ~0.1 μm–10 μm in the early stages of formation. Hence an alternative method to Transverse Micro-Radiography for the analysis of erosive lesions, having superior sensitivity to mineral content changes within the enamel surface and performed on commonly available laboratory equipment, would be of value. The desirable features of any potential method are the ability to quantify mineral content from the surface through to the bulk region of the enamel with sub-micron spatial resolution in the x, y and z directions. No less desirable are the cost and timeliness of the analysis. The combination of these features in a single method would furnish the ability to measure the effects of demineralisation from caries or erosive processes and the influence of emerging and established active agents on the demineralisation- remineralisation equilibrium.

One possible technique is Scanning Electron Microscopy in Back-Scattered Emission mode (BSE-SEM) which has been employed to investigate the mineral content of bone tissue (Bloebaum, Skedros, Vajda, Bachus, & Constantz, 1997; Roschger, Fratzl, Eschberger, & Klaushofer, 1998; Skedros, Bloebaum, Bachus, & Boyce, 1993). The technique is readily accessible and provides the potentially attractive benefits of sub-micron spatial resolution and rapid analysis.

In a BSE-SEM image the intensity (or grey level) of an area is proportional to the mean atomic number ($\overline{z}$) of the area imaged. The $\overline{z}$ of a compound can be calculated using the formula:

$$\overline{z} = \sum (N_i A_i Z_i) / \sum N_i A_i$$

where N_i is the number of atoms of an element $_i$ in the compound with atomic weight A_i and atomic number Z_i (Lloyd, 1987).

In studies carried out on bone it has been shown that when there is a single mineral phase present of relatively high $\overline{Z}$ in an organic matrix then $\overline{Z}$ is proportional to the mineral content in Wt% (Skedros et al., 1993). This has been exploited to quantify mineral distribution in histological sections of bone using BSE-SEM image analysis (Bloebaum et al., 1997; Roschger et al., 1998). Work carried out on dentine has also correlated the mineral content quantified by BSE-SEM analysis to micro-hardness data on carious dentine samples (Angker, Nockolds, Swain, & Kilpatrick, 2004). Since tooth enamel contains a relatively homogeneous defective hydroxyapatite (HA) mineral phase, similar to bone and dentine (Junqueira, 2003), it is proposed that this method of quantification should be applicable to tooth enamel.

This paper describes the assessment and development of a routine method for assessing the mineral content of lesioned tooth enamel over a wide depth range i.e. to include the early stages of acid erosion up to extensive caries formation. By comparing BSE-SEM data from a range of caries and erosive demineralised samples directly with results from both Transverse Micro-Radiography and micro-hardness measurements respectively, the potential and validity of the BSE-SEM approach will be illustrated.

2. Materials and methods

2.1. Initial sample preparation

Bovine incisors were initially autoclaved at 121 °C and stored in saturated thymol solution until required. Blocks of sound enamel were prepared from these specimens using a previously described polishing and preparation routine (Lynch et al., 2011).

2.2. Caries lesions

The caries lesions samples used in this study were a sub-set of those utilised in Lynch et al., 2011. From an original set of 120 samples, ten samples were selected to represent two groups of n = 5 where the groups had different mineral distribution characteristics. To achieve these differing mineral characteristics in the caries lesions, blocks of sound bovine enamel were demineralised at 37 °C and pH 4.6 in either unsaturated (7d) or part-saturated (8d, 4.1 mM Ca^{2+}, 8 mM Pi) lactic acid /methyl cellulose gel system. These samples were subsequently analysed for mineral content using a previously described TMR method (Lynch et al., 2011).

2.3. Erosive lesions

Blocks of sound bovine enamel, supported in resin discs, were exposed to a citric acid challenge (1% citric acid, pH3.8, room temperature) for periods of 5, 10, 15 or 20 min with n = 10 samples per treatment group. Each individual sample had a region which had been protected from the acid treatment by the application of an acrylic lacquer mask. After acid treatment the lacquer was removed using acetone. N.B. this method permits retrospective crater depth measurement for the acid-eroded region if bulk surface loss occurs i.e. cross-sectional SEM imaging provides direct measurement of the height difference between the lacquer-protected reference region and the acid-eroded region. Subsequently, micro-hardness readings were taken (Vickers diamond, 1.9 N, 20 s) from the acid-treated and untreated reference areas of each sample.

2.4. BSE-SEM method

2.4.1. Selection of standards

As outlined above, the BSE-SEM method is based on the premise that the image intensity (or grey level) is directly proportional to the mean atomic number ($\overline{Z}$) of a single mineral phase and in turn $\overline{Z}$ is proportional to the mineral content in wt.%. For tooth enamel the single mineral phase is hydroxyapatite with a calculated $\overline{Z}$ of 14.06. The corresponding mineral content value from the literature for pure hydroxyapatite is 96 wt.%. (Ross & Pawlina, 2006). Hence for studies of lesioned tooth enamel it is necessary to have standards for calibration of BSE-SEM signal which cover as broad a range of $\overline{Z}$ values as possible. The criteria used for selection of standards has been their availability as pure materials, inorganic in nature and readily polishable to a smooth finish when embedded in epoxy resin. The following five mineral standards were selected :-

Magnesium fluoride ($\overline{Z}$ 10.17), Alumina ($\overline{Z}$ 10.65), Magnesium ($\overline{Z}$ 12.0),

Aluminium ($\overline{Z}$ 13.0) and Silicon ($\overline{Z}$ 14.0)

In addition, the surrounding resin area provides a sixth reference since it can be assumed to have a mean atomic number of 6.0.

2.4.2. Preparation of standards and lesioned enamel samples

The five pure mineral standards were embedded in a single epoxy resin block using Struers Epofix mounting resin and Struers Epofix hardener, pre-mixed immediately before use in the volume ratio of 15 :

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