A new technique for analyzing trace element uptake by human enamel

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Fluorine (F) and strontium (Sr) are key elements in the de- and remineralization of teeth. To quantitatively analyze the distribution of F and Sr, micro-particle-induced gamma/X-ray emission (PIGE/PIXE) technique was used. The cavities were prepared and filled with the fluoride- and Sr-containing restorative materials (FSCMs) in extracted human molars. The single-section enamel specimens were prepared by slicing from the buccal to lingual surface including the FSCMs. After 5 weeks of automatic pH cycling, the demineralization was calculated by integrated mineral loss (ΔIML) from transverse-microradiography. The distributions of F and Sr were analyzed by the PIGE/PIXE technique. The micro-PIGE/PIXE technique indicated a fluorine uptake difference between the enamel surface and enamel cavity wall. ΔIML of FSCMs were significantly lower than intact enamel. The micro-PIGE/PIXE technique enables measurement of F and Sr uptake from FSCMs into enamel, which would be beneficial for research on caries development and prevention.

Keywords: Strontium, PIXE, Automatic pH cycling, Caries

INTRODUCTION

Many epidemiological studies have suggested the anticarious effect of Sr1-3, which can replace calcium (Ca) in hydroxyapatite because of their physicochemical similarity4-6). To elucidate the mechanisms of the Sr effect, many in vitro studies about Sr incorporation into hydroxyapatite have been performed. Dedhiya et al.7) indicated the formation of Ca-Sr apatite surface complex on synthetic hydroxyapatite under conditions resembling cariogenic attacks, and suggested the surface complex could govern the driving force of the dissolution reaction. The other studies reported Sr incorporation can slow down hydroxyapatite crystal growth8) and stabilize hydroxyapatite precursor phases9). Sr, therefore, can increase the number of biological nucleation sites10). In addition, it was reported nucleation of Sr substituted hydroxyapatite is easier than that of hydroxyapatite, and therefore Sr substituted hydroxyapatite can act as a template for hydroxyapatite growth11). Furthermore, incorporation of Sr into hydroxyapatite crystal causes an expansion of the crystallite, therefore elements with smaller radii can be incorporated into hydroxyapatite12). Especially, Sr and F in combination improved the crystallinity of carbonated hydroxyapatite much more than Sr or F alone13,14). These results suggest synergistic effect between Sr and F for low carbonated hydroxyapatite which is very similar to enamel to decrease the acid reactivity15). Despite of these studies, conclusive mechanism of Sr anticarious effect is unclear16).

Several restorative materials such as conventional type glass ionomer cements or resin composites containing fluoride and Sr (FSCMs) are assumed to enable uptake of fluoride (F) and Sr17-19) by dental hard tissue. These elements contained in filled FSCMs are incorporated into enamel as precipitation of eluted elements into enamel surface and/or direct uptake by enamel cavity wall, and affect the de- and remineralization of the enamel20,21). To evaluate uptake and the anticarious effects of these elements, their distributions in dental hard tissues has not yet been established. In previous studies22-24), to evaluate the Sr concentration in dental hard tissue, whole enamel and/or dentin were dissolved with acid. The amount of Sr dissolved in the acid was measured; however, this technique does not allow evaluation of the Sr distribution. In other studies, stepwise grinding25), multiple etching26,27) and tooth biopsies28) were used to analyze the Sr distribution in human teeth. With these techniques, the obtained data were at the mm level. However, a technique that can analyze distributions at the µm level is ideal. Ngo et al.29) examined F and Sr uptake in human dentin by electron probe microanalysis (EPMA) and reported that the minimum detection limit of Sr by EPMA was 0.05 wt%. This threshold seems inadequate because the average Sr concentration in human enamel is approximately 100 ppm22-24). In addition, EPMA requires sample pretreatment steps such as predrying and vapor deposition processing, which can cause shrinkage or cracks.

To overcome these difficulties, we adopted an analysis with particle-induced gamma/X-ray emission (PIGE/PIXE), which is a technique in which a target is bombarded with accelerated cations. Qualitative and quantitative analyses of elements on the target surface are possible by measuring the discharged gamma rays or X-rays30). This multi-elemental, nondestructive technique has excellent sensitivity across a wide range
of atomic numbers\textsuperscript{31}. Its detection limit is the parts-per-million range\textsuperscript{30}. Because no pretreatment is required and analysis is performed in air, the method may be useful to detect trace elements without damaging samples\textsuperscript{30,32}. PIXE detects characteristic X-rays from elements with more than 2 electron shells, whereas relatively light elements such as F are detected with PIGE\textsuperscript{33,34,35}.

In previous studies, the distribution of F around fluoride-containing materials was mapped and F uptake by human enamel was analyzed by PIGE/PIXE\textsuperscript{20,21}, which could provide quantitative distribution data at the µm level (micro PIGE/PIXE system) in the air. In this in vitro study, we aimed to establish a technique to analyze the Sr and F uptake by human enamel using the micro PIGE/PIXE system.

**MATERIALS AND METHODS**

Experimental procedure had four phases as following; 1) specimen preparation and automatic pH cycling, 2) evaluation of mineral loss and inhibition of demineralization, 3) micro PIGE/PIXE analysis, and 4) statistical analysis.

**Specimen preparation and automatic pH cycling**

This experiment was approved by the Research Ethics Committee of Hokkaido University Graduate School of Dental Medicine (approval number 2013-02). The outermost layer of 20 noncaries extracted human molars was removed with 2 N perchloric acid for 1 min at 20°C, to resolve the individual differences of F and Sr contained in each tooth\textsuperscript{36}. These teeth were stored in deionized water at 5°C before use. On the buccal sides of 10 teeth and palatal sides of the other 10 teeth, cavities were prepared with a diamond point (Shofu diamond point FG #301, Shofu, Inc., Kyoto, Japan) and air turbine with pouring water. Each cavity was with the width of approximately 1.0 mm and the depth of approximately 1.5 mm. Two following materials were used as a source of Sr and F: either conventional type glass ionomer cement (EX group; Fuji IX GP Extra, Lot. No 1112221, GC Corporation, Tokyo, Japan) or cavity varnish (PRG group; PRG Barrier Coat, Lot. No 0411, Shofu, Inc., Kyoto, Japan). The cavity varnish was injected into the cavity with a dental explorer. Ten teeth were restored for each group (5 buccal and 5 palatal cavities). The other side without restoration was used as a control.

The teeth specimens were then cut into 200-µm-thick longitudinal sections perpendicularly to the buccal surface including the materials, and the sections were ground to approximately 150-µm thickness by using whetstone #1000 (king deluxe whetstone 1000, Matsunaga stone corporation, Osaka, Japan) and #2000 (ceramic whetstone M15, Shapton corporation, Tochigi, Japan) with water cooling. These sections were then separated into experimental sides with fillings and control sides without fillings. All of the surfaces except for the buccal and palatal sides were covered with sticky wax. Transmission electron microscopy (TEM) grids were halved and fixed to the control sides for superimposition on images obtained by transverse microradiography (TMR).

To simulate daily acid attack in the oral cavity, pH cycling (pH 4.5–6.8) was performed for 5 weeks in an automatic pH-cycling system\textsuperscript{36}. The demineralizing solution (pH 4.5) contained 0.2 M lactic acid, 3.0 mM calcium chloride (CaCl\textsubscript{2}), and 1.8 mM potassium dihydrogen phosphate (KH\textsubscript{2}PO\textsubscript{4}); the remineralizing solution (pH 6.8) comprised 0.02 M 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 3.0 mM calcium chloride (CaCl\textsubscript{2}), and 1.8 mM KH\textsubscript{2}PO\textsubscript{4}. At 8:00 AM, 11:00 AM, 2:00 PM, 5:00 PM, 8:00 PM, and 11:00 PM, pH changes were induced by flowing the demineralizing solution at a rate of 50 mL/min for 2 min.

**Evaluation of mineral loss and inhibition of demineralization**

TMR images of each section were examined before and after pH cycling according to a previously described procedure\textsuperscript{37}. In brief, microradiographs were obtained in a soft X-ray system (CSM-2, Softex Corporation, Kanagawa, Japan) by using an aluminum step wedge and high-resolution photo plates (HRP-SN-2, Konica Minolta, Inc., Tokyo, Japan). For quantitative analysis of the same area in serial images of each restored side, the interface of the FSCM and the cavity wall was superimposed. The TEM grid was superimposed for the controls. In three regions (150, 250, and 350 µm from the restorative material or TEM grid), the average mineral density of 50-µm\textsuperscript{2} areas at 0.67-µm increments was measured\textsuperscript{38}. Integrated mineral loss (ΔIML) was calculated from the mineral profiles obtained before and after pH cycling\textsuperscript{39}.

The rate of dissolution inhibition (RDI) was calculated as follows:

\[
\text{RDI} = \frac{(\text{ΔIML}_c - \text{ΔIML}_s)}{\text{ΔIML}_s},
\]

where ΔIML\textsubscript{c} and ΔIML\textsubscript{s} represent the integrated mineral loss of the restored and control sides, respectively.

**Micro PIGE/PIXE analysis**

Micro-PIXE/PIXE analysis was performed as previously described\textsuperscript{20,21}. In brief, a 3.0-MeV proton beam was emitted from an ion microbeam apparatus (Fig. 1(a)). Each sample was attached directly to the window at the end of the microbeam system\textsuperscript{38}. The beam spot was approximately 1 µm in diameter and the beam current was approximately 100 pA. The sample was bombarded by the proton beam in ambient air. The maximum scanned area was 1,000 µm\textsuperscript{2}. A nuclear reaction (i.e., \textsuperscript{19}F (p,γ)\textsuperscript{20}F) was used to measure the F concentration; the generated gamma rays were detected with an 81-cm\textsuperscript{2} sodium iodide detector, which was placed 5 mm behind the sample. Ca and Sr concentrations were measured by micro-PIXE, which was simultaneously performed with a silicon lithium detector in vacuum\textsuperscript{38}. The beam intensity was monitored according to the X-ray yield from a copper (Cu) foil for quantitative analysis\textsuperscript{38}.

Quantitative analysis of trace elements in PIGE/
PIXE is performed based on the counts of characteristic gamma/X-rays discharged from specimens. To quantitatively analyze F and/or Sr using the PIXE system, standard materials are needed to convert the gamma/X-ray counts into F and/or Sr concentrations. Quantitative analyses of Ca and F were performed as previously described\(^2\). Sr standard materials were prepared by pressing and forming hydroxyapatite with different concentrations of strontium carbonate (\(n=5\); containing 0, 1.0, 1.5, 2.0, and 2.5 g strontium carbonate). The mass concentrations of Sr in these mixtures were analyzed by X-ray fluorescence (XRF). The mixture with 0 g of strontium carbonate was analyzed once, and the others were analyzed thrice. In cases of nonuniformity, the micro-PIXE-analyzed area was changed. Five uniform areas in each standard material were examined. The standard curve for Sr was created by comparing the mass concentration of Sr and the Sr/Cu ratio from the micro-PIXE analysis. Plots of Sr mass concentration versus Sr/Cu count were linearized with commercial software (Microsoft Excel 2010, Microsoft Corporation, Redmond, WA, USA).

Each tooth specimen was analyzed by using the aforementioned micro-PIGE/PIXE technique. Figure 1(b) shows representative mappings of Ca, Sr, and F in a specimen. Then quantitative analysis was performed by using the standard curves for each element. F and Sr distributions were evaluated in areas of enamel cavity wall or enamel surface. The interfaces of enamel surface and enamel cavity wall were determined as previously explained\(^2\). The accumulated amounts of F and Sr in four different regions, the enamel surface of the EX (EXS) and PRG (PRGS) and the enamel cavity wall of the EX (EXW) and the PRG (PRGW), were calculated (Fig. 1). The concentrations of F and Sr in the FSCMs were also analyzed by the micro-PIGE/PIXE technique.

**Statistical analysis**
The paired t-test and t-test were used to compare ΔIML and RDI, respectively \((p<0.05)\). The Games-Howell procedure was used to compare the distribution and accumulated amounts of F and Sr between the groups \((p<0.05)\).

The concentrations of F and Sr in the FSCMs were compared by the t-test \((p<0.05)\).

**RESULTS**
Figure 2(a) shows the average ΔIML of the restored and control sides. The restored sides had significantly lower ΔIML. Furthermore, ΔIML was significantly lower in the EX group than in the PRG group. As shown in Fig. 2(b), the EX group had significantly higher RDI.

Figure 3 shows the standard curve for Sr. A significant positive correlation \((R^2>0.95)\) was found between the Sr mass concentration and the Sr/Cu count.

Higher concentrations of F and Sr were detected near the enamel surface and FSCMs than in the deeper regions. The EX group tended to show higher concentrations of these elements than the PRG group. In both groups, the enamel surface tended to show a higher F concentration than the cavity wall, whereas the cavity wall tended to show a higher Sr concentration than the enamel surface (Figs. 4(a) and (b)). The amount of F that accumulated at the EXS and EXW was significantly larger than at the PRGW (Fig. 4(c)). Further, Sr accumulation was significantly greater at the EXS and EXW than at the PRGS.

The F and Sr concentrations were \(6.4\times10^4\pm4.2\times10^4\) and \(3.8\times10^3\pm2.3\times10^3\) ppm in the EX and \(3.8\times10^3\pm3.0\times10^3\) ppm in the PRG.
Fig. 2 Standard curve of strontium (Sr).
Sr mass concentration and Sr/copper (Cu) count were determined by X-ray fluorescence (XRF) and micro-particle-induced X-ray emission (micro-PIXE), respectively.

Fig. 3 Average mineral loss (ΔIML) after 5 weeks of automatic pH cycling (a) and rate of dissolution inhibition (RDI). The rate of dissolution inhibition (RDI) was calculated as follows: (ΔIMLc − ΔIMLs)/ΔIMLc, where ΔIMLc and ΔIMLs represent the integrated mineral loss of the restored and control sides, respectively. (b) The glass ionomer cement (EX) and cavity varnish (PRG) groups. The vertical lines indicate the standard deviation. *Significant difference by the paired t-test or t-test (p<0.05).

Fig. 4 Concentrations of (a) fluorine (F), (b) strontium (Sr) and (c) their accumulated amounts per 43 µm² in different regions of the glass ionomer cement (EX) and cavity varnish (PRG) groups. The vertical lines represent the standard deviation. *Significant difference by the ANOVA test and the Games-Howell test (p<0.05).

ppm and $8.3\times10^2\pm7.5\times10^2$ ppm in the PRG, respectively. The EX showed significantly higher F and Sr concentrations than the PRG.

DISCUSSION

The restored sides showed significantly lower mineral loss than the controls. F and Sr uptake in the enamel appeared to inhibit enamel dissolution around the FSCMs. The significantly higher RDI in the EX group may be explained by the finding that higher F uptake decreases mineral loss in enamel\( ^{20} \).

These results suggested that F and Sr distribution in enamel could be detected and quantitatively analyzed by the PIGE/PIXE system in this study. The Sr distribution showed a gradual transition in areas deeper than 43 µm. In contrast, the F distribution varied until the depth of 118 µm. F seems to penetrate enamel more deeply than Sr. The higher concentrations of Sr and F at the enamel surface and enamel cavity wall of the EX group may be attributed to the high F and Sr concentrations in the FSCM. In both groups, F accumulation was greater at the enamel surface, whereas Sr accumulation was greater at the enamel...
cavity wall. FSCMs release a greater amount of F in acidic conditions than in neutral conditions, but Sr release has a different tendency. F is easily eluted in acidic conditions with a high concentration of hydrogen cations, as it is an anion. At neutral pH, the F released into solution will be taken up by hydroxyapatite because of the electric charge difference. The results are also attributable to the properties of each element. F is more diffusent in solution than Sr because it has a smaller ionic radius. Furthermore, F possibly has a tendency to be absorbed into enamel through the enamel surface rather than the enamel cavity wall, whereas Sr has the opposite tendency.

In previous studies regarding F uptake by human enamel, fluorideapatite samples were used as F standard materials (Ca_{10}(PO_4)_6(OH)_{2-x}F_{x}, where x=0, 0.25, 0.5, 0.75, or 1.0). When Sr is substituted for Ca in hydroxyapatite, the Sr/Ca ratio varies to a greater extent than the F/Ca ratio of fluoroapatite. Therefore, Sr-substituted hydroxyapatite is difficult to refine with the desired Sr concentration. In the present study, mixtures of hydroxyapatite and strontium carbonate were used as Sr standard materials. The Sr mass concentration calculated by XRF and the Sr/Cu count determined by micro-PIXE showed a significant positive correlation, which indicates that mixtures of hydroxyapatite with this Sr compound could be used for PIXE-based quantitative analysis of Sr.

Quantitative analysis of trace elements using PIXE is reportedly affected by the matrix composition, especially for thick targets. Therefore, standard samples should have matrices as similar as possible to those of the specimen (in this case, human enamel). Use of hydroxyapatite as the matrix for the standard sample decreased the “matrix effect” compared with use of the National Institute of Standards and Technology (NIST) bone ash standard, which was used as the standard sample in a previous PIXE analysis of trace elements in human teeth.

The standard materials with a higher Sr content showed a wider range of Sr/Cu counts during micro-PIXE. A higher Sr content may prevent uniform mixing of hydroxyapatite and strontium carbonate. This problem was managed by analyzing only uniform areas in each material. The preparation method for standard materials should, however, be improved.

The detection limit of Sr in the present micro-PIXE analysis (<50 ppm) was much lower than that with EPMA. A beam spot as small as approximately 1 μm was previously shown to enable high-resolution analysis of the elemental distribution. Furthermore, no cracks or shrinkage of the specimens occurred because they were analyzed in air without pretreatment. In addition, comparison of the F and Sr distributions in each specimen was possible because of their simultaneous analysis. These features of micro-PIGE/PIXE may be useful to analyze many types of elements incorporated into dental hard tissues, if appropriate standard materials are available. Therefore, micro-PIGE/PIXE may be a suitable technique for studying caries development and prevention.

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REFERENCES


