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A Microbeam Small-Angle X-ray Scattering Study on Enamel Crystallites in Subsurface Lesion

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Abstract. The early caries lesion in bovine tooth enamel was studied by two different X-ray diffraction systems at the SPring-8 third generation synchrotron radiation facility. Both allowed us simultaneous measurement of the small and large angle regions. The beam size was 6µm at BL40XU and 50µm at BL45XU. The small-angle scattering from voids in the hydroxyapatite crystallites and the wide-angle diffraction from the hydroxyapatite crystals were observed simultaneously. At BL40XU an X-ray image intensifier was used for the small-angle and a CMOS flatpanel detector for the large-angle region. At BL45XU, a large-area CCD detector was used to cover both regions. A linear microbeam scan at BL40XU showed a detailed distribution of voids and crystals and made it possible to examine the structural details in the lesion. The two-dimensional scan at BL45XU showed distribution of voids and crystals in a wider region in the enamel. The simultaneous small- and wide-angle measurement with a microbeam is a powerful tool to elucidate the mechanisms of demineralization and remineralization in the early caries lesion.

1. Introduction

Early caries lesion in tooth enamel is caused by acids produced by bacteria in dental plaque and dentally observed as a opaque white spot. In a cross section along the direction perpendicular to the enamel surface, a caries lesion appears as an enamel decay with a relatively intact surface layer and a mineral loss in the subsurface region [1]. When not treated, the decay spreads into the dentin and forms a cavity. Over the past three decades, many studies have been made on demineralization of the early subsurface lesion under different conditions. Most of these studies used transversal microradiography (TMR) [2] to measure the mineral content. The TMR analysis measures the mineral density from microradiography of thin sections of enamel and is regarded as the standard method to evaluate the degree of demineralization [3,4]. However, since evidence on the crystal structure of hydroxyapatite (HAp), which comprises tooth enamel, cannot be obtained by this method, the apparent change in mineral content could be partially due to changes in other substances such as precipitates of calcium phosphate. The enamel HAp is arranged in rod-like units called crystallites. The morphology of HAp crystallites was studied by high-resolution transmission electron microscopy and decrease in

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the HAp crystal size by demineralization was reported [5]. Such morphological changes in the submicron order are expected to change small-angle X-ray scattering (SAXS).

In the present study, we used SAXS with a microbeam to investigate the HAp crystallites in the subsurface lesion [6]. Wide-angle X-ray diffraction (WAXD) from HAp was recorded simultaneously. A 6µm microbeam was small enough to study the 100µm thick subsurface lesion and enabled us to compare the crystallite structure with the TMR results at the spatial resolution required for the analysis of the lesion. In another experiment, use of a 50µm beam enabled us to map two-dimensionally the HAp crystallites and the voids in them. In addition, we suggest the possibility of using laboratory equipments for similar studies.

2. Materials and Methods

2.1. Preparation of artificial subsurface lesion

Bovine incisors were obtained from a slaughterhouse (Osaka, Japan). Enamel blocks (approx. 7 mm × 7 mm × 3 mm depth) were cut out (Figure 1a) and embedded in resin (GC CORP, Japan). The blocks were polished with a wet polishing paper (3M wrapping paper 800, 1000 and 2000 grid, USA) to expose a sound and flat enamel surface. Then, sound and demineralized zones were made on the same enamel surface as follows. First, half of each block surface was covered with a nail varnish as described [7]. Then, subsurface lesions were formed by demineralization in a two-layer system of 8% methyl cellulose gel (Fluka, USA) and 0.1 M lactate buffer (pH 4.6) at 37°C for 14 days [4]. After lesions were made in the demineralized zone by the acid treatment, the nail varnish was washed off with acetone to reveal the intact, sound zone.

After the demineralization treatment, 150µm–thick enamel slices were cut out from the middle of each block perpendicularly to the surface with a water-cooled diamond-coated wire saw (Well Diamond Wire Saws Co., Ltd, Germany) (Figures 1a, 1b).

2.2. Simultaneous WAXD and SAXS measurement at BL40XU

The experiments were carried out at the BL40XU beamline (Figure 2) [8] of the SPring-8 third-generation synchrotron radiation facility with an X-ray energy of 15.0 keV (bandwidth about 3%). An X-ray microbeam was obtained by placing a 5µm collimating pinhole on the focused beam [9]. The beam expanded to about 6 µm at the sample because of Fresnel diffraction. A guard pinhole with a diameter of 200µm was placed in front of the sample. The distance between the two pinholes was about 80 mm (with a small ion chamber between them). To remove the scatter from the edge of the collimating pinhole, it is necessary to make this distance as large as possible. On the other hand, the beam becomes larger with distance from the collimating pinhole because of diffraction. Thus, the distance of 80 mm was chosen as a compromise in the current setup. The X-ray flux was about 3×10^{11} photons/sec. The X-ray detector for SAXS consisted of an X-ray image intensifier (V5445P,
Hamamatsu Photonics, Hamamatsu, Japan) [10] coupled with a tandem lens to a cooled CCD camera (ORAC-II-ER, Hamamatsu Photonics). The WAXD pattern was recorded with a CMOS flatpanel detector (C9728DK, Hamamatsu Photonics) [11]. An ionization chamber (S-1329, Oken, Tokyo, Japan) was also inserted between the flatpanel detector and the vacuum tube to measure the transmission of the X-rays through the sample. The distance from the sample to the SAXS detector was about 2m and to the WAXD detector about 100mm. The reciprocal spacing was calibrated with a powder diffraction pattern of LaB$_6$ for WAXD and the meridional reflections from dried chicken tendon collagen (1st order at 63 nm) for SAXS. The SAXS and WAXD patterns were recorded simultaneously. The reciprocal spacing is expressed as $q = 4\pi \sin \theta / \lambda$, where $\theta$ is the scattering angle and $\lambda$ is the wavelength of the X-ray.

An enamel section was mounted vertically so that its enamel edge was more or less horizontal (Figure 1b). The X-ray beam passed perpendicularly through the slice. The sample was moved upwards with a 5µm step so that the X-ray beam scanned across the enamel from the surface towards dentin. At each step, SAXS and WAXD patterns were recorded. The region to the depth of 150 µm was scanned. The sound and demineralized zones of each sample were studied.

2.3. Simultaneous WAXRD and SAXS measurement at BL45XU

BL45XU has tandem helical undulators as an X-ray source and uses a diamond crystal as a beam splitter [12]. In the present experiment, the protein crystallography hutch with three sets of diamond double-crystal monochromators was used. One set of the monochromators was used to obtain a 13.8 keV X-ray. A bent cylindrical mirror was used to focus the X-ray beam. The beam size without a pinhole was approximately 250 µm × 250µm. A 0.1mm thick tantalum pinhole with a diameter of 50 µm was inserted at 335 mm upstream of the specimen (Figure 3a). The sample-to-the-detector distance was 450 mm. At 15mm upstream to the specimen was a guard pinhole with a diameter of 0.8 mm. With a beam stop with a diameter of 2 mm, the scatters from the edge of the pinhole fell within the beam stop. Thus, the insertion of the 50µm pinhole did not affect the background in the small-angle region significantly. The flux through the 50µm pinhole was about $3 \times 10^9$ cps. The X-ray detector was Jupiter210 (RIGAKU, Tokyo, Japan), which comprises of four CCDs with tapered fibers. The detection area was 200 mm × 200 mm. The pixel size was 100 µm × 100 µm with 2×2 binning. In order to cover a wide q-range, the detector was moved so that the beam stop is located near the bottom of the detection area (Figure 3b). A fiber diffraction pattern from a dried chicken tendon collagen (Figure 3c) shows the 3rd-order meridional reflection at about 1/21 nm$^{-1}$ and the meridional reflection at 1/0.28 nm$^{-1}$ (Figure 3b) showing that this setup covers a q-range of about two orders of magnitude. Since the SAXS intensity exceeds the WAXD intensity by nearly three orders of magnitude, the software for the measurement control was designed to allow multiple exposures with different exposure time. Thus, images with different exposure times were recorded from the same spot in the sample to cover the large intensity range.
3. Results

3.1 Small-angle X-ray scattering (SAXS)

The SAXS patterns from the sound enamel showed a weak equatorial scatter, that is, at right angles to the c-axis of the HAp crystallites, which is the long axis of the needle-shaped crystallites (Figure 4a). These patterns often showed superposition of two sets of diffraction diagrams with different orientations, which were given by two regions with different crystallites orientations [13]. The scatter is likely to be due to the density difference between the needle-shaped HAp crystallites and the matrix between them that is composed of lighter non-mineral substances including water and carbonate. In the demineralized zone, the equatorial scatter was greatly enhanced in the region 30-60 µm from the surface (Figure 4d). Since it has been known that decalcification by acid treatment creates longitudinal voids in the matrix regions by reducing the size of the HAp crystallites [14], the SAXS enhancement is interpreted to be due to formation of voids in the enamel. This scatter from voids is similar to the SAXS from carbon fiber [15] and cellulose [16]. The SAXS from a powdered enamel sample was interpreted in the same manner [17]. Although markedly enhanced, the general shape of the equatorial streak was unchanged, showing that the general texture of the apatite was unchanged by demineralization. The equatorial scatter is rather featureless and hard to interpret. If the voids can be assumed to be cylindrical with different diameters, detailed analysis can be made. However, since the voids seen in electron micrographs are all different in size and shape, it is hard to use standard software for the analysis. Qualitatively, the scattering profile from the demineralized zone is more steep in the small-angle region (q < 0.2 nm\(^{-1}\)) than that from the sound zone, suggesting that there are a population of larger voids in the demineralized zone (Figure 5a). If the voids are assumed to be

![Figure 3. The SAXS/WAXD recording at the BL45XU protein crystallography hutch. (a) Arrangement in the experimental hutch. A 50µm pinhole and a 800µm pinhole were placed at 335 mm and 15 mm upstream of the specimen, respectively. Helium paths were employed to avoid attenuation of the beam by air. The area detector (Jupiter210, RIGAKU, Tokyo, Japan) was placed off-center to cover a larger q-range. (b) A wide-angle diffraction pattern from collagen (dried chicken tendon). The strong arc at the top is the 0.28nm meridional reflection. The black lines are unusable regions between segments of the CCDs. (c) A small-angle diffraction pattern from collagen. The peak just outside of the beam stop (pointed by an arrow) is the third order meridional reflection from the 63nm periodicity.](image)
infinitely long cylinders parallel to the long axis of the crystallites, the intensity along the equator is proportional to \((J_1(qR)/(qR))^2\) (where \(R\) is the radius of the cylinder), which asymptotically decreases with \(q^3\) in the large \(qR\) region (Figure 5b). After the Lorenz factor, the observed intensity is expected to decrease with \(q^4\). The slopes of the curves (Figure 5a) are much less steeper than expected. This may imply that there is contribution from region with \(qR < 3\) in Figure 5b, suggesting the presence of rather small voids with diameters of tens of nm. However, in electron micrographs [14], the voids are very irregular in the cross-sections and too close to each other to diffract independently. These factors must be affecting the scattering intensity and hamper more precise interpretation.

Figure 4. Diffraction patterns and intensity profiles from bovine tooth enamel. (a) SAXS pattern recorded by an X-ray image intensifier. The two arrows indicate the directions of the equators in the two crystalline domains. (b) WAXD pattern recorded by a CMOS flat panel detector. The arrow indicates the (100) equatorial reflection. (c) Absorption measured by an ion chamber along the direction perpendicular to the enamel surface. (d) Integrated intensity in the small-angle region. (e) Integrated intensity of the (100) equatorial reflection. In (c)-(d), SOUND indicates the data obtained from the intact region and DEM that from the acid-treated (demineralized) region.
3.2 Wide angle X-ray diffraction (WAXD)

The WAXD pattern from a slice of bovine enamel shows diffraction from the HAp crystallites (Figure 3a). The HAp microcrystals (crystallites) in the enamel are 0.05-0.2 µm in diameter and up to a few µm in length [18,19]. The long axis corresponds to the c-axis of the unit cell of the HAp crystal. The crystallites are arranged with their c-axes nearly parallel to each other but their rotation around the c-axis is random. Thus, the WAXD pattern from the enamel resembles that of fibrous materials and the diffraction spots can be indexed on a hexagonal three-dimensional lattice [20]. The diffraction spots are arced because the crystallites are not pointing to the same direction throughout the path of the X-ray beam in the sample. However, the widths across the arcs in the radial direction are similar to the X-ray energy spread (about 3%), suggesting that the perfection in each crystallite is high. In most samples, the c-axis of the crystallite that is, the meridian) was inclined by 30-60 degrees from the surface of the enamel that was more or less parallel to the surface of the tooth. Although difficult to observe in the partial pattern in Figure 3b, as was the case with the SAXS, two sets of diffraction diagrams with different orientations were often superimposed, with the c-axes tilted by about 90 degrees from each other ("two fiber axes" patterns) [13]. In reciprocal space, the rings of the equatorial reflections always intercept the Ewald sphere unless the c-axis becomes nearly parallel to the X-ray beam. On the other hand, the meridional reflections are spots in reciprocal space and easily deviate from the Ewald sphere when the c-axis is tilted. Thus, the intensity of the equatorial reflections is much less affected by the tilt of the c-axis and gives a more reliable measure of the amount of HAp crystals. In the present study, the two-dimensionally integrated intensity of the (100) equatorial reflection (at a Bragg spacing of 0.815 nm) after background subtraction was measured as an index of the amount of the HAp crystallites.

When the integrated intensity of the (100) peak was plotted against the distance of the X-ray beam from the surface, it was almost constant in the sound zone but lower within about 100 µm below the surface in the demineralized zone (Figure 4e). This result is similar to that by TMR. The radial widths (full-width at the half-maximum) of the (100) and (002) peaks were 0.0006 and 0.0021 nm\(^{-1}\), respectively, in the control enamel. Within the resolution of the present study (the width is determined

\[ \text{Figure 5. (a) Intensity profiles of the small-angle scattering from bovine enamel. The thin and thick lines are from the sound and the demineralized zones in enamel, respectively. The profiles were obtained by integrating along the arc. (b) Calculated scattering intensity profile from a solid cylinder. R represents the radius of the cylinder.} \]
mostly by the energy bandwidth as explained above), these values did not change significantly along the depth or by demineralization (data not shown).

3.3 SAXS/WAXD measurements
In some experiments the SAXS and the WAXD were measured simultaneously (Figure 2). In this case, the WAXD was measured with a CMOS flatpanel detector [11] which could record only less than a half of the diffraction pattern (Figure 4b). However, since the direction of the equator was usually confined to the azimuth of about 45 degrees from the surface, by adjusting the orientation of the detector, it was possible to monitor the intensity of one (100) diffraction spot (Figure 4e). A simultaneous measurement of X-ray transmission through the sample with an ionization chamber (Figure 4c) is equivalent to TMR. The SAXS data were obtained at about 3m downstream with an X-image intensifier and a CCD camera (Figure 4d). This simultaneous measurement ensures that the transmission, SAXS and WAXD data are obtained at the identical spot of the same sample, facilitating comparison of these data.

As exactly the same part of the sample was interrogated by the three techniques, two interesting features can be pointed out. One is the surface layer with higher density that is clearly seen with TMR [1]. This is less distinct in the absorption (Figure 4c) because of the lack of spatial resolution that is apparent in the gradual rise of absorption at the edge. Although the FWHM of the microbeam is about 6 μm, there is a considerably long tail due to Fresnel fringes from the pinhole. Also, the enamel sample may not be set up with its surface edge exactly parallel to the X-ray beam. Compared with the absorption, the surface layer has even lower intensity in the WAXD data (Figure 4e). This may show that the surface layer contains a lower amount of the HAp crystallites per density, but a study at higher spatial resolution will be required to confirm this.

The other interesting observation is that, compared with the absorption and the SAXS intensity, the WAXD intensity in the demineralized region seems to return to the sound level in the shallower region: the WAXD intensity recovers at around 80 μm, while the changes in the absorption and the SAXS intensity persist to more than 100 μm. It can be also noticed that, up to 80 μm from the surface, the decrease of absorption in the demineralized zone is larger than the decrease of the WAXD intensity. These are discussed in detail below.
3.4 2D mapping of the SAXS and WAXD

At BL45XU, by scanning a sample with the 50-µm pinhole, two dimensional maps of the SAXS and WAXD intensity were created. The SAXS map (Figure 7b) shows the integrated intensity in q=0.35-3.2 nm\(^{-1}\) summed in all directions. In the demineralized region, the surface of the enamel showed much stronger SAXS than in the sound zone. The width of the high intensity region is about 200 µm, wider than that measured with a 6µm beam, because of the 50µm beam size in this experiment. The WAXD map (Figure 7a) was created from the integrated intensity of the (100) reflection after background stripping.

Although not striking, compared with the SAXS map it is apparent that the surface edge in the demineralized zone has receded slightly. This is due to the lower intensity in this region (Figure 4e). In other areas, there is a gradual intensity difference which may represent changes in the thickness of the slice.

Figure 6. An X-ray diffraction pattern from bovine sound enamel recorded by a large CCD detector (Jupiter210) at BL45XU. (a) The entire diffraction pattern. (b) An enlarged image of the small-angle region.
4. Discussion

4.1 SAXS/WAXD measurements at the two beamlines

Two different types of the SAXS/WARD microbeam measurement were performed on the tooth enamel with sound and demineralized regions. The measurement at BL40XU employed a 6 µm beam and used different detectors for the small and wide angle regions. On the other hand, at BL45XU the beam was 50 µm and a single detector was used to record both small and wide angles. The former method can resolve the detailed profile of the subsurface lesion which extends only about 100 µm below the surface. The latter can be used to study the global differences of SAXS and WAXD in a large area. One advantage of the microbeam at BL40XU is its high flux, above $10^{12}$ cps with the 6µm beam which was made possible by the low energy resolution (about 2%). On the other hand, BL45XU is monochromatic (energy resolution about than 0.01%) and hence the diffraction peaks are sharper. Although this is not very important in the present study in which only integrated intensity was measured, it can be vital when one is trying to measure the width of a peak precisely.

The simultaneous SAXS/WAXD measurement is a useful technique when relating structures at different scales. In the present study on the subsurface lesion, the demineralized WAXD profile was different from the sound profile only within 100 µm from the surface, while it extended deeper in the SAXS profile. This comparison is only possible when the two profiles are obtained from exactly the same part of the sample. These results suggest that the materials in the enamel other than the HAp contribute significantly to the absorption and the SAXS. The matrix between the crystallites is filled with non-crystalline materials such as carbohydrates, lipids and proteins. Loss of these materials decreases absorption, and the voids due to the loss may give rise to small-angle scattering without affecting the diffraction from the HAp.

It is common to use two detectors to record the small and wide angle regions of the scattering [21]. However, the part of the scattering that can be recorded by the wide angle detector is usually limited to either linear or a part of the two-dimensional scattering. Thus, it is ideal to use a large detector that can cover a large q-range. This has been realized at BL40B2 at SPring-8 where an image plate

![Figure 7. Results from the 2D scan with the 50µm beam (horizontally 0.02mm×56points, vertically 0.2mm×56points). The region below the nick is the demineralized zone. Note the difference in the scales in horizontal and vertical directions.](image-url)
detector with an area of 30cm × 30cm (RIGAKU RAXIS-VII) is used to cover a q-range of 0.35 to 18 nm⁻¹ [22]. The detector used at BL45XU has a smaller area (20cm × 20 cm) and thus the beam stop had to be located near an edge to cover a wide q-range. However, since this detector has a faster readout than the image plate detector, it was possible to do a 2D scan. An ideal detector is a CCD-based detector with a larger area such as ADSC Quantum-315 or MAR MX-325 which has an area larger than 30cm × 30cm. A large detector area will enable us to use a longer camera length and improve the small-angle resolution. If a 2D scan is not required, use of a larger but slower detector can be considered: for example, RIGAKU RAXIS-V has a detection area of 40cm × 40cm [23]. Two technical points should be reminded in a SAXS/WAXD experiment with a single detector: (1) the size of the exit window of the vacuum tube becomes larger for a larger detector. In the cameras described here, 130µm thick Kapton film is used as a window material. A special care should be taken to make a window as large as 40cm × 40cm. (2) A larger detector size means a large data size. The amount of data created by a 2D scan could become challenging.

4.3 Future prospects
Subsurface lesion is quite common and it may be possible to reverse the process before a cavity is formed. In fact, it has been suggested that saliva may have such a function: Ca and phosphate in saliva may be able to remineralize the lesion [24]. Chewing gums which are claimed to facilitate this process are in the market. It is anticipated that the remineralization rebuilds the HAp crystals that are lost by acids. However, the remineralization may be due to accumulation of precipitates of calcium phosphate. The present X-ray method confirms that the mineralization definitely involves increase of the HAp crystals. Thus, it will be a powerful tool to examine caries lesion in future. In this context, it should be pointed out that the study can be made to some extent with a laboratory X-ray apparatus. In order to evaluate the degree of demineralization and remineralization, a linear scan with a beam size of 50µm may be sufficient as evident in Figure 7. An X-ray tube with a linear focus smaller than 50µm is available with a reasonable flux. It should be noted that for a linear scan the beam does not have to be point-collimated. A linearly collimated beam may distort the 2D diffraction pattern. However, it may be used for the purpose of evaluating SAXS because measurement of total scattering intensity suffices. Also, as the (100) reflection is an isolated peak, a short linear beam does not affect the reliability of the WAXD result. Although the synchrotron microbeam experiment is ideal to obtain the most reliable data, a laboratory-based method can make it a more general tool in dentistry.

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