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Nanostructure of healthy and caries-affected human teeth

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Abstract

Spatially resolved small-angle x-ray scattering based on synchrotron radiation combines the quantitative assessment of nanometer-sized components using scattering with the real-space imaging by means of scanning. The method enables us to study the effect of caries-induced damages on the inorganic and organic nanoscopic components in human teeth. We demonstrate for several 200- to 500-μm-thin tooth slices that the bacterial processes dissolve the ceramic components in enamel and dentin, but the dentinal collagen network remains practically unaffected with respect to its abundance and orientation in early stages of caries and in parts of extended carious lesions. Consequently, we speculate that future caries treatments can be developed reversing the effect of bacterial attacks by means of suitable remineralization of the dentin.

From the Clinical Editor: In this groundbreaking study of caries pathology using synchrotron-based X-ray scattering, the authors demonstrated that while bacterial processes do dissolve the ceramic components in enamel and dentin; however, the dentinal collagen network remains unaffected, enabling the development of future caries treatments that re-mineralize the dentin.

Key words: Tooth nanostructure; Dentin; Dentinal collagen; Small-angle X-ray scattering; Synchrotron radiation

The hierarchical structure of the hard tissues of human teeth guarantees function for decades. Caries, the most frequent disease, is known to damage the enamel, the dentin, and the cementum through the production of acidic species that dissolve the ceramic tooth components as also observed in the dental erosion that habitually accompanies excessive citrus fruit consumption.[1] Unfortunately, the destroyed tooth structures do not fully regenerate, although remineralization of small carious lesions occurs under optimized dental hygiene.[2]

The treatment of carious lesions is nowadays accompanied by the removal of affected hard tissues. The progression of caries is enhanced in the dentin in comparison with the progression in the enamel. The faster spread through the dentin is generally visible in sections or projections as triangles with their basis along the enamel-dentin junction.[3] Therefore, dentists are regularly forced to amputate not only the massively damaged enamel, but also a significant amount of healthy enamel to guarantee the complete substitution of carious dentin by the restoration material. As the current restoration materials do not fully fit the performance of the natural healthy hard tissues, the resulting limited lifespan renders further interventions necessary.[4,5] The dentin not only consists of ceramic components, but also of about 20 wt% organic material.[6] It has been hypothesized that the organic components, in particular the collagen, may remain unaffected. This hypothesis is based on electron microscopy inside carious lesions, which proves the strong similarity between collagen in selected healthy and caries-affected regions.[7] Furthermore, it has been speculated that the remaining collagen network can provide nucleation sites for remineralization.[8,9] To prove the hypothesis an imaging method has to be identified that allows comparing the collagen abundance in healthy and caries-affected parts of a tooth. Spatially resolved small-angle x-ray scattering (SAXS) is a technique that enables the identification of collagen using its 67-nm periodicity[10] over extended areas with resolution in the micrometer range, i.e., about 40,000 scattering patterns per square centimeter.[11] Because the patterns are average values of the illuminated volume along the x-ray beam, the contributions from the surfaces are negligible and the potential influence of artifacts from cutting several 100-μm-thick slices is small. Consequently, scanning SAXS is well suited for the structural characterization of tooth slices over the entire nanometer range.

Methods

Eight teeth, five carious and three healthy, were extracted for clinical reasons and cut into 200- to 500-μm-thin slices

No comflict of interest is reported by the authors.

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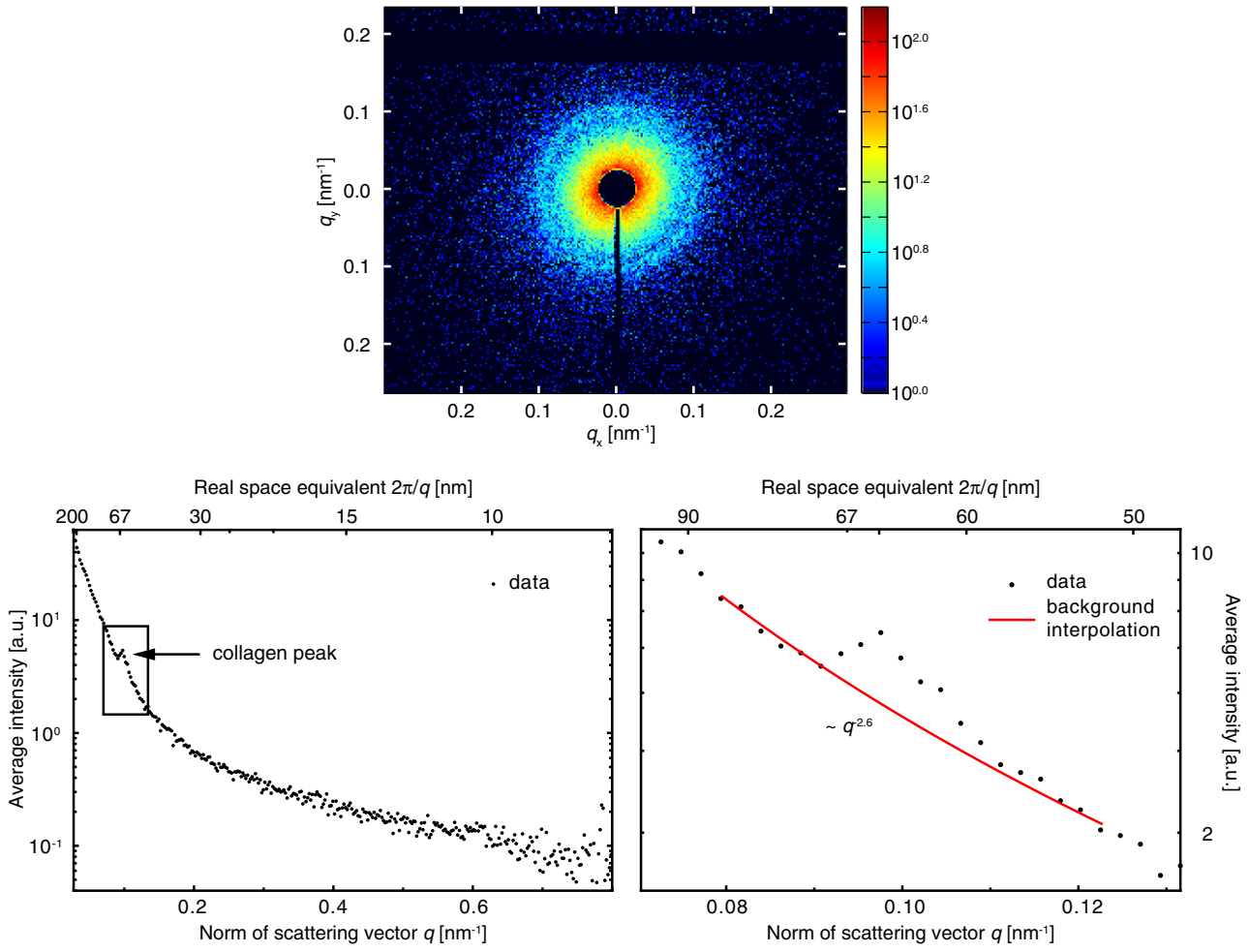

Figure 1. A typical SAXS pattern of healthy dentin with collagen ring is given on the top row. On the bottom panels, the radial-integrated intensity is shown. The collagen-associated peak can be extracted subtracting the background indicated by a solid line in the bottom right panel.

parallel to the tooth's long axis with a diamond band saw (Exakt Apparatebau GmbH, Norderstedt, Germany). The slices of the teeth were stored in phosphate-buffered saline (PBS). Only one slice from the healthy teeth was stored in air.

Synchrotron radiation-based micro computed tomography (SRμCT) measurements were performed at the beamline W 2 (HASYLAB at DESY, Hamburg, Germany) operated by the HZG-Research Center, Geesthacht, Germany, in standard absorption contrast mode[12] in two sessions. One carious specimen was measured using a photon energy of 45 keV. The 1440 projections acquired with asymmetric rotation axis equally distributed along 360 degrees with a pixel size of 4.6 μm were combined[13] and binned by a factor of 2 to improve the density resolution.[14] The spatial resolution of 8.8 μm was determined from the 10% value of the modulation transfer function of a strongly x-ray absorbing edge.[15] The remaining three carious specimens were also measured using a photon energy of 45 keV, but with 1800 projections over 360 degrees and a pixel size of 3.14 μm, and a spatial resolution of 8.4 μm. Prior to reconstruction, the data were binned by a factor of 4.

Scanning SAXS measurements were performed at the cSAXS beamline of the Swiss Light Source (PSI, Villigen, Switzerland).[11] The 18.58 keV photon beam was focused to 8 × 25 μm^2 at the position of the specimen for the raster scans. The specimens were scanned through the beam in 50 μm steps in *x*- and *y*-directions, perpendicular to the beam. Two modules of the PILATUS 2M detector[16] positioned at 7.1 m from the specimen were used to record the scattering patterns. One carious and two healthy specimens were scanned with 20 msec exposure time per frame. The remaining specimens were measured with an exposure time of 180 msec with 24 modules of the PILATUS detector in use. Specimen-to-detector distance was determined with the first scattering order of a silver behenate calibration specimen. Prior to scattering pattern acquisition, the attenuated through-beam was recorded with the same step-size, 8 msec exposure time per point without beam stop and with attenuated beam. To reduce air scattering, an evacuated flight tube was placed between specimen and detection unit.

Results

The organic ingredients of dentin contain mainly collagen-I fibrils. The building blocks of collagen arrange themselves along the collagen fibril with a main periodicity of 67 nm, as determined, for example, by means of atomic force microscopy and SAXS.[17,18]

Figure 1 shows, in the top row, a typical scattering pattern from healthy dentin and the corresponding radial-integrated intensity (q-plot). A distinct peak, associated with collagen-I, is found at the q-values between 0.091 and 0.111 nm^{-1}, corresponding to a real space periodicity of 69.2 to 56.5 nm. The base intensity below the peak can be approximated with a power-law with an exponent of -2.6 (see Figure 1 diagram bottom right). The intensity above the fitted curve is solely associated with the scattering signal from the collagen.

Perfectly disc-shaped structures yield a scattering intensity, which decays according to a power-law with an exponent of -2.[19] It is known that dentin contains mainly plate-like crystallites, especially near the dentin–enamel junction (DEJ).[10,20,21] Considering the deviation of dentin crystallite shapes from perfect discs and the presence of the organic phase, the exponent of -2.6 chosen for the collagen extraction is a reasonable estimate.

Carious infections are known to reduce the degree of calcification in tooth hard tissue. To visualize this phenomenon in quantitative fashion, two x-ray absorption measurements have been performed. Figure 2 (top) shows the logarithm of the x-ray transmission, corrected for specimen thickness, measured in line with the SAXS scan, which illustrates the expected differences between dentin and enamel. The carious lesion can be clearly identified as dark region in the center of the dentin. It is clearer in tomography data as the result of improved contrast. Therefore, a slice of the tomography data is represented in the center of Figure 2. The carious lesion appears as darker region in the center of the dentin and in the enamel due to the reduced degree of calcification. The effect is prominent and clearly detectable in the related x-ray absorption histogram given in Figure 2 below the images. Here, the carious dentin appears as shoulder and tail on the left side of the healthy dentin peak.

The images in Figure 3 (left and middle columns, top images) show the elastic scattering towards angles, which correspond to nanostructures between 56.5 and 69.2 nm for the total and the collagen-related signal, respectively. Dentin and enamel exhibit different scattering potentials and are therefore distinguishable. We found it surprising that the caries-affected regions appear brighter in enamel and dentin; i.e., they yield a stronger signal in comparison with that of the healthy tissues. This behavior can be attributed to a change in mean crystallite size of caries-affected hard tissues,[21] or to an increased porosity in the affected dentin. The collagen-related scattering signal (bottom images), extracted from the SAXS patterns of the same tooth slice, does not allow any discrimination between healthy and caries-affected parts in the tooth, where the degree of demineralization amounts to 10%. The intensity for the enamel is close to zero because of the negligible amount of organic species in enamel.[6] The dentin, however, contains anisotropic, heterogeneously distributed nanostructures as visible in both images. The scattering intensity near the dentin-enamel junction is more than a factor of two higher in comparison with the

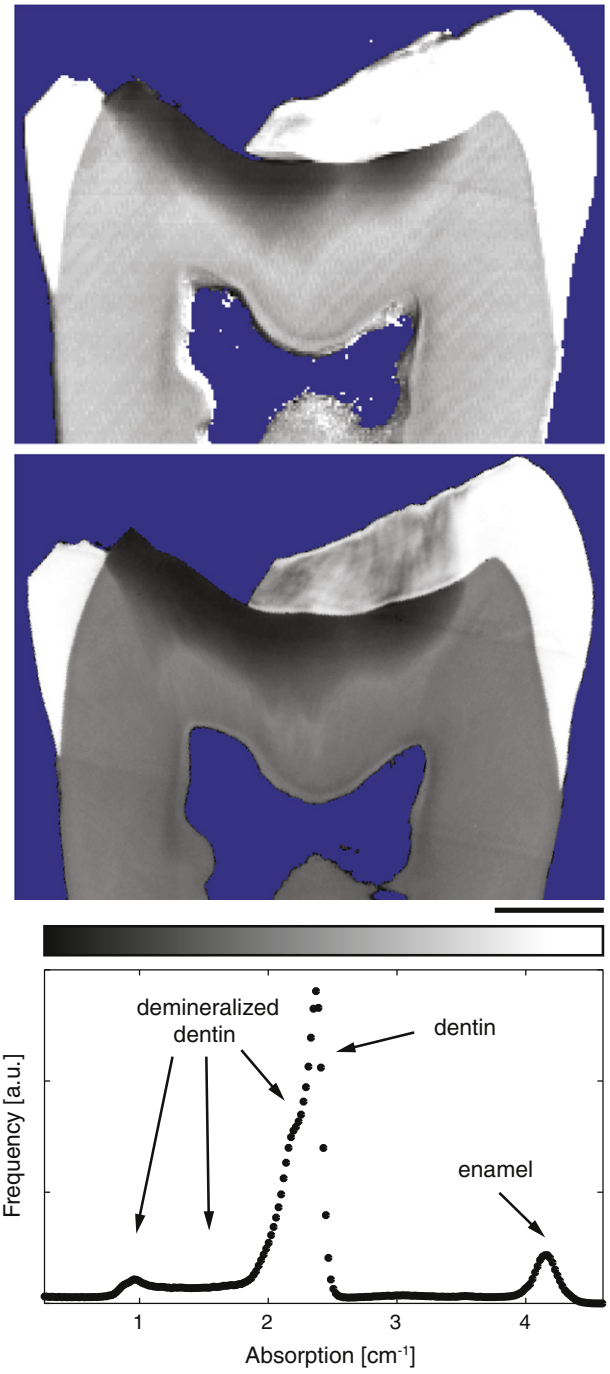

Figure 2. On top, the transmission data from the SAXS measurements of one carious specimen are presented (sam05). In the middle, a slice through the tomography data of the same specimen is shown. The carious regions are identifiable as darker regions in both enamel and dentin. The length of the bar corresponds to 2 mm. The x-ray absorption histogram of the entire tomography data is given below. The missing enamel is the result of a preparation artifact.

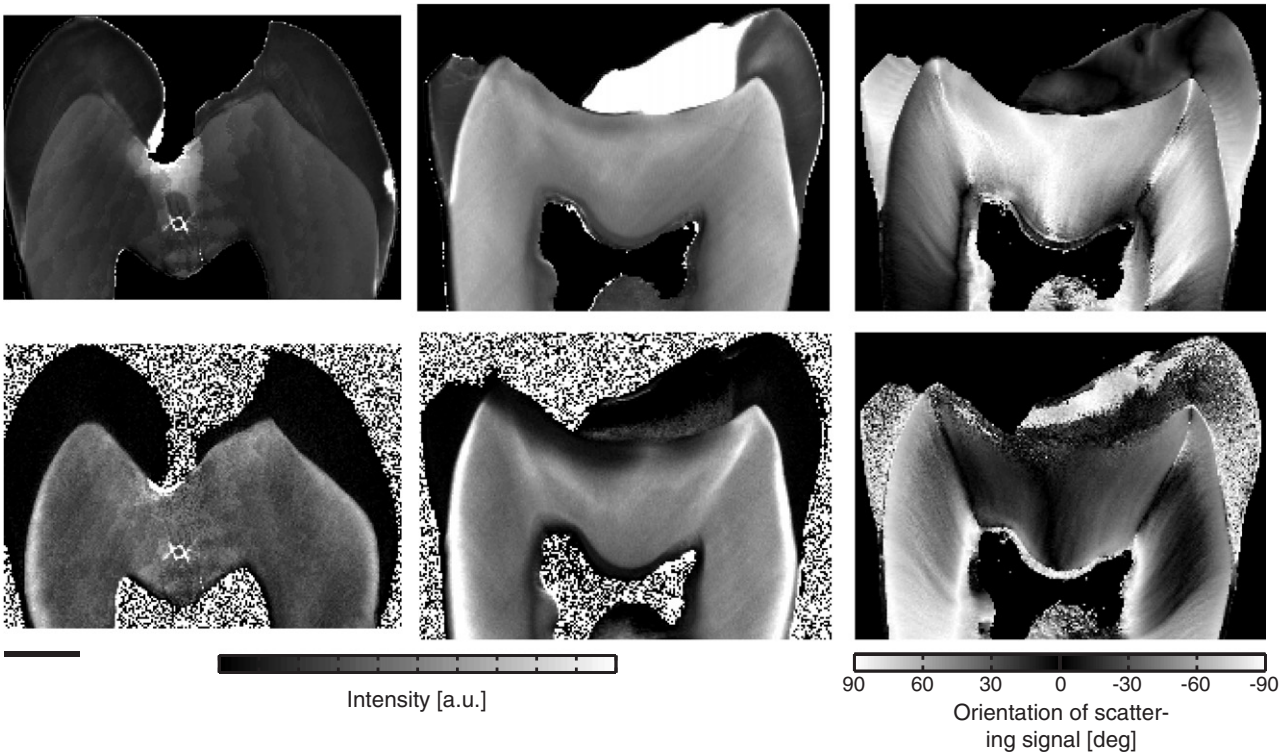

Figure 3. The first two columns of images display the elastic scattering towards angles, which correspond to nanostructures between 56.5 and 69.2 nm, for all features (top) and the collagen-related signal (bottom), respectively (sam13 and sam05). The scattered intensity is represented in average counts per pixel, corrected for specimen absorption and thickness. The caries-affected region in the dentin can be clearly identified from the integral signal, while only parts of it can be seen in the collagen-related part. The images in the right column show the preferential orientation of the nanostructures between 56.5 and 69.2 nm in sam05. The gray value labels the angle between the horizontal direction (black) and the preferential orientation of the scattering intensity. The length bar corresponds to 2 mm.

regions near pulp. This phenomenon is better illustrated in Figure 4, which includes line plots through the collagen-related signal (solid line) and the absorption (dashed line) through healthy and caries-affected regions. The collagen signal of the carious region does not differ from the one of the healthy region, if the x-ray absorption is within 70% of that of healthy tissue; i.e., the onset of collagen deterioration seems to be significantly delayed with respect to the decomposition of the mineral phase.

The analysis of the SAXS patterns does not only allow analyzing the scattering potential but also assessing the preferential orientations of nanostructures. The average direction of a scattering signal in a selected range of nanostructures (q-range) was determined approximating the azimuthal intensity distribution by a cosine.[11] The preferential orientation of the scattering signal caused by the tooth's nanostructures between 56.5 and 69.2 nm determined from such a fitting procedure is given in Figure 3, right column – total signal on top and collagen-related signal on bottom. The collagen-related scattering signal is oriented along collagen fibrils, whereas the scattering signal related to ceramic components is typically oriented perpendicular to structures like rods and platelets. This allows deducing the orientation of nanostructures in the tooth from the orientation of the scattering signal as shown in Figure 3. The orientation of nanostructures changes at the dentin-enamel junction, being more or less perpendicular in the enamel and dentin at this position. In dentin one can clearly distinguish between an inner region around the pulp with vertically oriented hydroxyapatite crystallites and collagen fibers and an outer region with mainly horizontally oriented nanostructures. The carious lesion is only distinguishable in the topmost layer of the caries in the collagen orientation signal, but it is not seen in the total signal; i.e., the caries did not affect the average orientation of nanostructures. Figure 5, first and second columns, combines the information on abundance and orientation of the selected nanostructures for the total (first column) and the collagen-related (second column) scattering signals in four selected slices of three healthy teeth. The scattered intensity is coded as brightness. The color represents the preferred orientation according to the color wheel. For example, cyan marks mainly vertical scattering, red marks horizontal scattering, etc. The color saturation displays the degree of orientation, which is defined as the ratio between oriented and total mean scattered intensities.[11,22] Enamel and dentin are clearly distinguished because of their different scattering potential and nanostructure texture. Similarities between different teeth and different positions inside one tooth concerning the organization of collagen and inorganic components can be seen (top and bottom row of Figure 5 respectively). Overall perpendicular

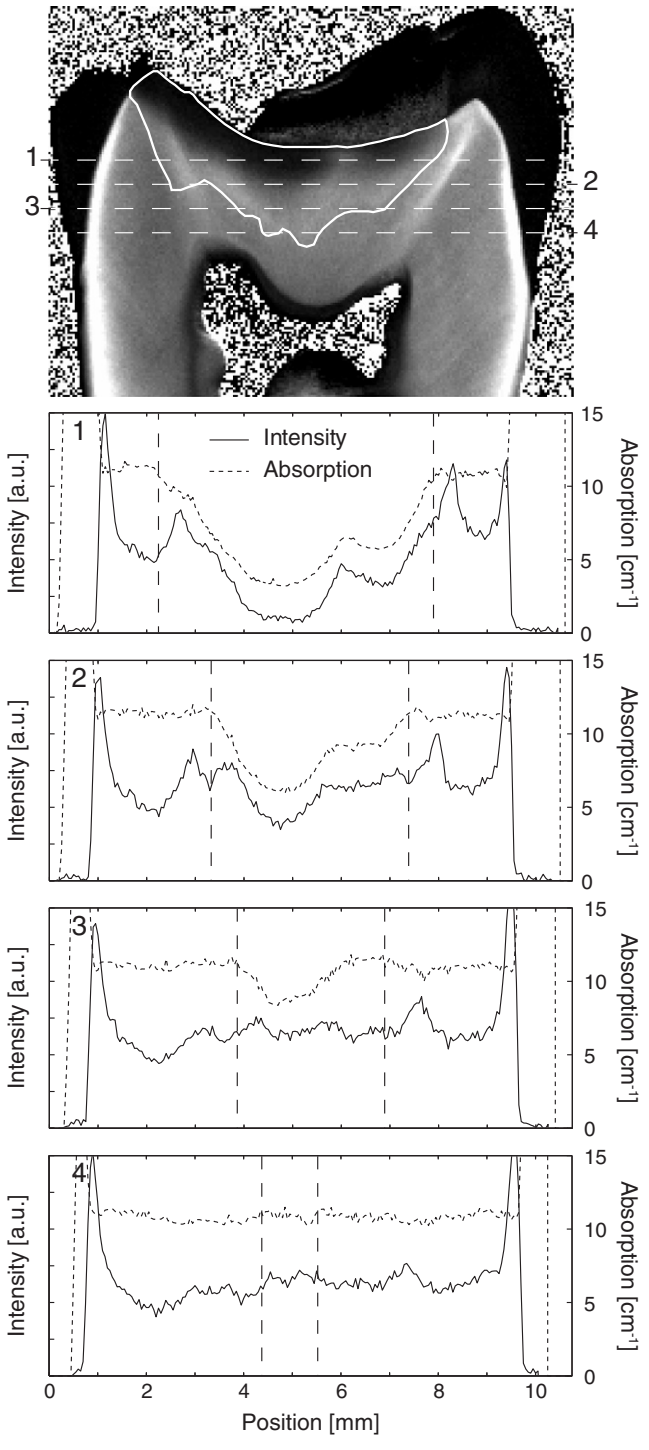

Figure 4. Intensity of the collagen-related signal across one carious tooth slice (sam05): The line plots according to the dashed lines in the image quantitatively show the changes in collagen scattering signal intensity (solid line) and specimen absorption (dotted line). The higher concentration of collagen fibrils near the dentin-enamel junction produces the prominent feature.

resp. parallel orientation of the scattering signal with respect to the dentin-enamel junction (DEJ) in dentin resp. enamel is found in all specimens. Sharp changes in nanostructure orientation, especially in the enamel and the collagen, are present on imaginary lines connecting the tooth cusps to the pulp. Figure 5, third and fourth columns, shows these data from four slices through the carious molars. The carious regions in both dentin and enamel can be identified easily as they present stronger overall intensity in comparison with the healthy part. The overall orientation of the scattered collagen intensity is mainly perpendicular to that of the total scattering in the dentin, revealing the parallel alignment of the collagen fibrils with the inorganic crystallites, whereas no significant collagen intensity is found in the enamel. In the collagen-related signal, the carious region can be identified in the first, second, and third slice shown. However, it appears less extended than in the total scattering signal. The overall pattern in the deeper caries resembles that of healthy dentin. This implies that the caries bacteria attacks do not change the abundance and orientation of the collagen network until a certain degree of demineralization is reached.

To relate the degree of decalcification with the strength of the collagen signal, the dentin of each specimen was divided in three regions, namely sound dentin (SD), mildly carious dentin (MCD), corresponding to the tissue presenting between 90% and 70% of the x-ray absorption of healthy dentin in each specimen, and carious dentin (CD), corresponding to tissue presenting less than 70% of the x-ray absorption of the healthy part. The classification was performed through histogram analysis of the transmission data. To account for the different slice thicknesses and absorption potentials, the data were corrected by dividing the scattering signal by the slice thickness, obtained from the SRμCT measurements, and the x-ray transmission obtained in line with the SAXS measurements.

Even though the x-ray absorption is heterogeneous in healthy dentin, this classification is an appropriate choice for intensity-based segmentation, as illustrated in Figure 6. The mean collagen signal and x-ray absorption for each region and specimen are shown in Table 1; the errors correspond to the standard deviation.

Discussion

The scanning SAXS images of human teeth show similarities to polarization microscopy pictures obtained from tooth slices many decades ago.[23] The preparation of the tooth slices for polarization microscopy, however, is much more laborious. The slices have to be thin enough for the optical transmission and their surfaces have to be well prepared to avoid artifacts. Because the photon energy can be adapted to the slice thickness in scanning SAXS, much thicker tooth slices can be investigated, reducing the potential influence of inadequate surface preparation. Another advantage of SAXS with respect to polarization microscopy is the precise quantification of abundance and orientation of nanostructures including collagen fibrils. The lateral resolution of scanning SAXS, however, is much worse than in polarization microscopy. Consequently the two methods can be regarded as complementary rather than competing.

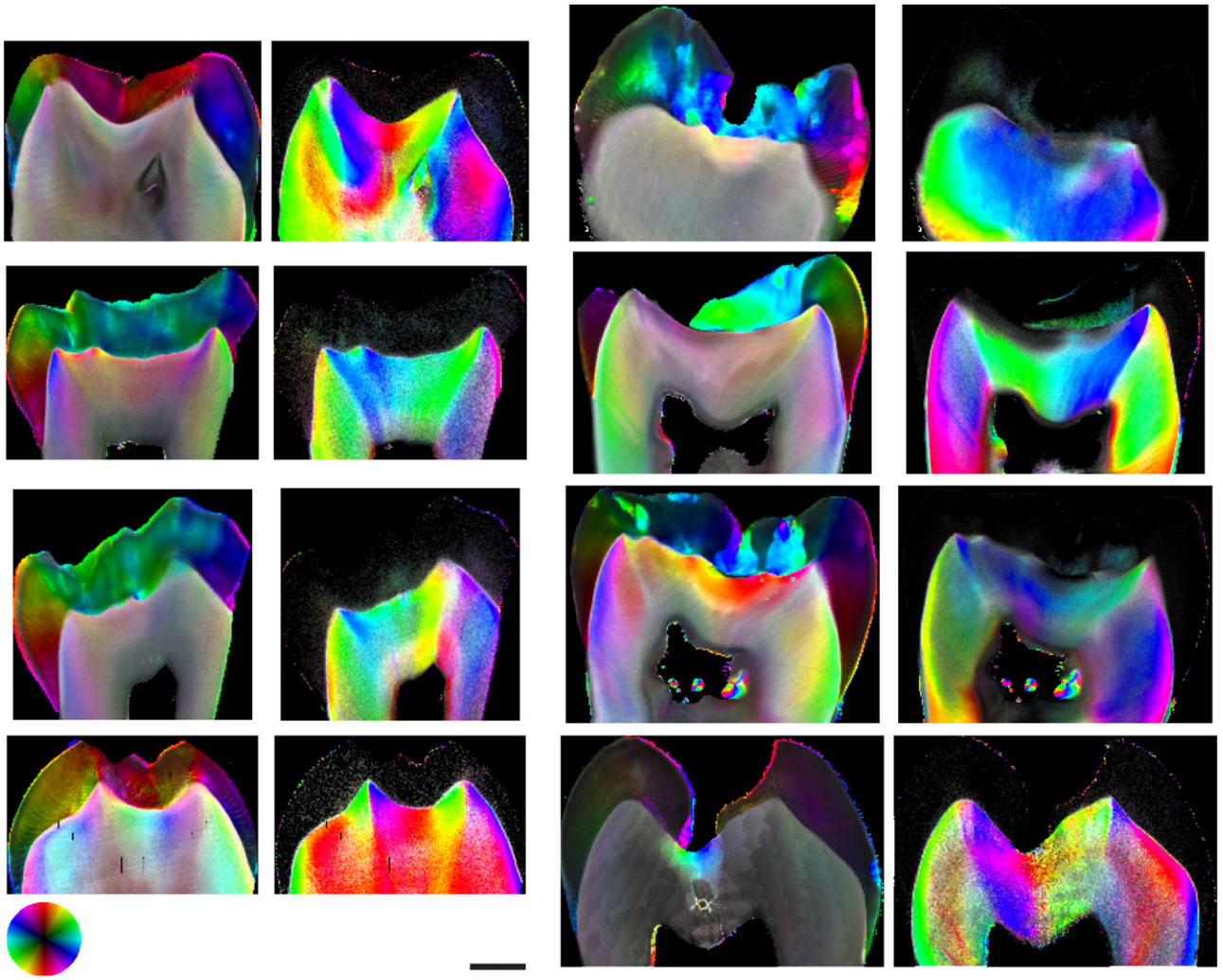

Figure 5. The color-coded images display four slices from three healthy (two left columns) and four carious (sam02, sam05, sam11, and sam13, two right columns) teeth. In the first and third columns, the total scattering signal corresponding to the range between 56.5 and 69.2 nm is given, whereas the second and fourth columns show just the collagen-related signal. The preferential orientations of the nanostructures of interest are color-coded according to the color wheel. The color intensity codes the scattered intensity, whereas the color saturation represents the degree of orientation. The length bar corresponds to 2 mm.

Caries bacteria destroy the tooth and degenerate the dentin. During the very early stages of caries, however, the nanometer-sized components are only slightly altered in size and orientation.[24] As opposed to local probe techniques, scattering measurements in scanning setup allow obtaining information on macroscopic specimen areas. In the range between 56.5 and 69.2 nm, the enamel exhibits a strong and anisotropic scattering signal caused by the ceramic components aligned roughly perpendicular to the dentin-enamel junction and almost perpendicular to the ceramic components within the dentin. Collagen does not exist in the enamel but exhibits within dentin a scattering signal perpendicular to the dominating calcium phosphate scatterers. The collagen is much better oriented than the inorganic stronger scatterers in the dentin. The nanostructures of the mineral phases of the enamel, however, are as well oriented as the collagen in the dentin. These observations are well in line with the well-established electron and polarization microscopy studies.[21,23]

In carious lesions, the mineral content is significantly reduced, as shown in countless radiographs. Because of the relative high cross-section of the minerals, one would expect a correspondingly lower scattering signal within the lesion in comparison with the surrounding healthy dentin. For the range between 56.5 and 69.2 nm, however, we detected a significantly higher scattering probability. The increase of the mean crystallite size in caries-affected dentin[21] can explain this phenomenon. Another explanation could be an increased porosity in dentinal tissue, similar to that found in enamel.[25] Independent of these explanations, however, we can state that this phenomenon allows identifying the carious lesion in the SAXS data, especially because the spatially resolved signals mark a clear border between carious and healthy dentin as verified by the SRμCT data.

The collagen-related signals allow only partially differentiating between carious and healthy dentin in the affected

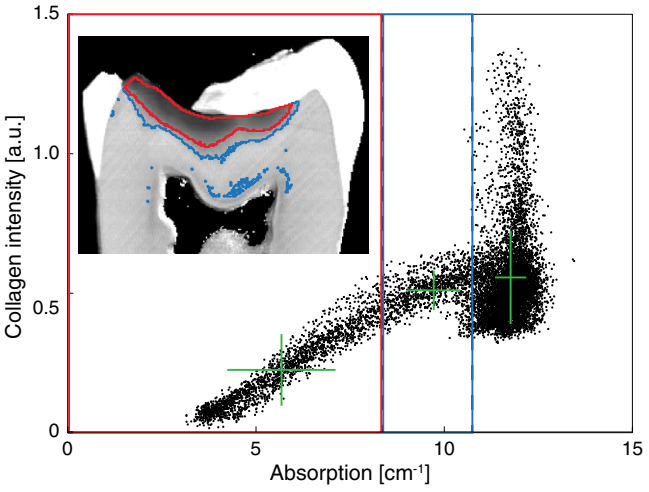

Figure 6. The plot shows the relation between collagen-related scattering signal and x-ray absorption in sam05. The blue- and red-colored regions correspond to MCD and CD. The green crosses correspond to mean values and standard deviation of each point cloud.

specimen. The presence of zones presenting different properties in carious dentin is well known and has, for example, been demonstrated by staining methods.[7,26] More recently, Pugach et al[27] reported a characterization of staining-based zones with the assumption that staining relates to distinct microstructure characteristics. They distinguished between apparently normal dentin, nonstainable affected dentin, partially de-mineralized dentin (light pink staining), having a mineral content of 60% ± 14% in comparison with healthy dentin, and a more demineralized zone (pink staining) exhibiting a mineral content of 29% ± 8% in comparison with healthy dentin. However, characterization through staining alone may not allow differentiating remineralizable, demineralized dentin from that which can be removed only in a conventional dental treatment. The upper and lower threshold for the region termed MCD in this study were chosen based on the absorption histogram of the x-ray transmission measured in line with SAXS, with the upper threshold corresponding to 90% of the peak position of healthy dentin lying outside this peak.

Considering the projective nature of the technique, where the demineralization front may not lie parallel to the beam direction, and thus dentin with different degrees of demineralization may overlap, the lower bound is a conservative estimate of what can still be considered mildly demineralized tissue.

The fact that half of the inspected specimens present a collagen signal in the MCD zone within 90% of the signal of sound dentin indicates that a significant amount of collagen is retained under mild demineralization, in these specimens. Five of the six remaining specimens present values between 65% and 80%, suggesting that mild to considerable damage to the collagen network has happened.

It is noteworthy that differences in the degree of preservation of the collagen occur between specimens obtained from the same tooth. Thus, not only the total degree of decalcification, but also the location within the carious lesion, seems to play a role concerning the damage to the collagen network. In addition, the projective nature of two-dimensional scanning SAXS may lead to overlapping MCD and CD zones, so that a mixed signal is obtained.

Caries bacteria destroy the tooth and degenerate the dentin, but at least during the initial stages or only mild demineralization, a significant part of the collagen network is conserved in abundance and orientation. We hypothesize that dentin with mainly healthy collagen may be suitable for remineralization.

Acknowledgments

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Table 1
Mean collagen signal intensities and x-ray absorption values of healthy and carious human teeth including their standard deviations

Specimen		Sound dentin (SD)		Mildly carious dentin (MCD)		MCD/SD [%]	Carious dentin (CD)		CD/SD [%]
		Collagen signal [a.u.]	Absorption [cm^{-1}]	Collagen signal [a.u.]	Absorption [cm^{-1}]		Collagen signal [a.u.]	Absorption [cm^{-1}]	
28_72	sam02	0.83 ± 0.22	10.2 ± 0.4	0.41 ± 0.10	8.2 ± 0.6	49	0.28 ± 0.07	6.1 ± 0.8	34
28_72	sam03	0.60 ± 0.16	6.1 ± 0.2	0.39 ± 0.15	5.0 ± 0.4	65	0.25 ± 0.12	2.5 ± 1.4	42
48_63	sam04	0.55 ± 0.14	11.0 ± 0.4	0.54 ± 0.07	9.1 ± 0.7	98	0.27 ± 0.13	5.1 ± 1.5	49
48_63	sam05	0.56 ± 0.17	10.9 ± 0.4	0.51 ± 0.07	9.0 ± 0.7	91	0.23 ± 0.13	5.3 ± 1.3	41
48_63	sam06	0.55 ± 0.17	10.9 ± 0.4	0.53 ± 0.11	9.1 ± 0.7	96	0.18 ± 0.12	5.3 ± 1.4	33
18_66	sam07	0.65 ± 0.22	9.7 ± 0.7	0.62 ± 0.23	8.0 ± 0.5	95	0.11 ± 0.12	4.0 ± 1.6	17
18_66	sam08	0.66 ± 0.15	11.2 ± 0.4	0.44 ± 0.16	9.2 ± 0.7	67	0.10 ± 0.11	6.0 ± 1.5	15
48_66	sam09	0.60 ± 0.16	11.0 ± 0.5	0.44 ± 0.16	9.0 ± 0.7	73	0.16 ± 0.11	5.9 ± 1.8	27
48_66	sam10	0.62 ± 0.16	10.9 ± 0.4	0.46 ± 0.17	9.7 ± 0.7	74	0.14 ± 0.10	6.0 ± 1.3	23
48_66	sam11	0.63 ± 0.16	11.4 ± 0.4	0.57 ± 0.14	9.5 ± 0.6	90	0.25 ± 0.13	6.1 ± 1.8	40
48_66	sam12	0.57 ± 0.19	9.7 ± 0.6	0.52 ± 0.18	8.1 ± 0.5	91	0.47 ± 0.14	6.2 ± 0.4	82
SB3	sam13	0.21 ± 0.04	9.0 ± 0.9	0.17 ± 0.04	7.4 ± 0.5	80	0.07 ± 0.01	5.7 ± 0.1	33

The ratios relate to the collagen signal intensities. a.u., arbitrary units.

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