Applicability of ToF-SIMS for monitoring compositional changes in bone in a long-term animal model

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Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a well-established technique in material sciences but has not yet been widely explored for implementation in life sciences. Here, we demonstrate the applicability and advantages of ToF-SIMS analysis for the study of minerals and biomolecules in osseous tissue. The locally resolved analysis of fragment ions deriving from the sample surface enables imaging and differentiation of bone tissue and facilitates histology on non-stained cross sections. In a rat model, bilateral ovariectomy combined with either a multi-deficiency diet or steroid treatment was carried out to create osteoporotic conditions. We focused our study on the Ca content of the mineralized tissue and monitored its decline. Calcium mass images of cross sections show the progressive degenerative changes in the bone. We observed a decreased Ca concentration in the edge region of the trabeculae and a decline in the Ca/P ratio. Additionally, we focused on the non-mineralized matrix and identified fragment ions that are characteristic for the collagen matrix. We observed trabeculae with wide ranges of non-mineralized collagen for the diet group owing to an impaired mineralization process. Here, the advantage of coeval monitoring of collagen and minerals indicated an osteomalacic model rather than an osteoporotic one.

1. Introduction

For analysing osseous tissue, a wide variety of in vivo and ex vivo methods are well established and unquestionable in their ability to assess bone quality. Standard techniques such as dual-energy X-ray absorptiometry (DEXA) measurements, quantitative computed tomography and X-ray micro-tomography (μCT) are widely used to determine bone mineral density (BMD) [1–3]. BMD represents a calculated mean value of bone mineral content in a given area (usually given as area density in mg mm⁻²). Clinically, a reduced BMD assessed by X-ray absorptiometry is considered to be the first indicator of osteoporosis [1]. However, the BMD does not account for the bone structure and composition in detail [2–4].

A more significant and accurate fingerprint value for the characterization of bone quality is the bone mineral density distribution (BMDD), which was introduced by Roschger and co-workers [5,6]. The BMDD is determined by quantitative backscattered electron imaging and is validated for clinical use [7]. The macro- and micro-structure of bone can be determined non-destructively by using μCT with a lateral resolution in vitro of 10 μm [2,3,8]. Microbeam X-ray diffraction and the more recent synchrotron-based small and wide-angle X-ray scattering (SAXS and WAXS) are well-established diffraction methods for the structural and textural analysis of biological tissue such as bone with a spatial resolution of 1 μm [9,10]. Supplementary information includes the elemental analysis and mapping by X-ray fluorescence imaging (XRF) and μ-XRF for...
higher Z-elements. By means of focusing capillary optics, a spatial resolution of 10 µm can be achieved [11]. In 2010, the value of the combination of SAXS, WAXS and µ-XRF for the analysis of the structural and compositional properties of bone was successfully demonstrated by Mahamid et al. [12].

Furthermore, transmission electron microscopy (TEM) also provides structural information on the atomic level of small sample areas in vitro [13]. Raman microscopy and Fourier transform–infrared microspectroscopy (FT-IR) are used to characterize both the organic and inorganic matrix of bone with a spatial resolution of 1 and 10 µm, and FT-IR also provides information about the collagen cross-linking [14–16].

In addition, histochemical and immunohistochemical techniques such as stains and indicators have been optimized over decades and enable the identification of biological tissue, cell types and activity. However, staining and marker reagents are limited in number and combination, and the well-known compounds can be applied only in a targeted manner to visualize structures in biological tissues. Hence, each of the different methods provides essential information for bone characterization and is indispensable in clinical use (DEXA) or for biologists (histology).

To understand the complexity of the material composition better and to assess chemical and structural changes in diseased bone, methods are required that offer detailed chemical information and enable us to define tissue types, mineral content and distribution with high sensitivity and a high spatial resolution. In the present paper, we demonstrate that time-of-flight secondary ion mass spectrometry (ToF-SIMS) provides this information and that its supplementary use helps to bridge the gap between tissue and mineral analysis.

ToF-SIMS was originally developed as a technique for the analysis of inorganic solids and in particular for the evaluation of concentration differences with very high depth resolution. In recent years, several studies have been performed to explore and prove the capability of ToF-SIMS for surface analysis and structural investigations in biological samples [17]. The technique has been used to characterize phospholipids, peptides and other biomolecules on the cellular level [18–22]. The value of two-dimensional imaging as well as three-dimensional analytical reconstruction has been demonstrated [17]. In medicine, ToF-SIMS can be used to localize drugs and to explore their distribution in different types of tissue. Based on earlier studies on pure hydroxyapatite, for example, Malmberg et al. and Eriksson et al. characterized bone tissue by ToF-SIMS and analysed the incorporation behaviour of titanium implants [23–26]. Furthermore, ToF-SIMS studies on bone and implant materials have been published by Ni et al. [27] and Lu et al. [28]. In a more recent study, Zoehrer et al. [29] applied ToF-SIMS as a supplementary method to characterize human femoral bone. Up to now, ToF-SIMS has not been applied to study affected bone in a manner as detailed as we present herein.

In the following, we report systematic results on the use of ToF-SIMS mass spectra and images of high spatial resolution (less than 300 nm) for the analysis and characterization of diseased and normal bone in a long-term small animal rat model, where pathological changes are induced by ovariectomy combined with either a special multi-deficiency diet or a steroid medication. We reveal the advantages of ToF-SIMS using embedded but non-stained cross sections and use the chemical information to image and characterize simultaneously the inorganic and organic matrix.

The main objective of this paper is to demonstrate the wealth of information that we can obtain by using ToF-SIMS in addition to well-established techniques.

2. Results

2.1. Bone tissue analysis

2.1.1. Mass spectra

The procedure of ToF-SIMS analyses of bone samples carried out in our study is shown schematically in figure 1. A typical positive mass spectrum of trabecular area is depicted in
Figure 2. Positive ion mass spectra from mineralized bone tissue of a rat obtained with Bi⁺ primary ions. Mass spectra are shown in the ranges (a) 1 < m/z < 100, (b) 100 < m/z < 200, (c) 200 < m/z < 500, (d) 350 < m/z < 750. (e) Ca⁺ signal for the reference—12 months sham—and 12 months diet groups and (f) negative ion mass spectrum 1 < m/z < 100.

Figure 2a–d. Several fragment ions were found to be derived from a Ca₁₀(PO₄)₆(OH)₂ precursor species (ion, m/z value, in the following always given in Da: Ca⁺, 39.96; Ca₄PO₄⁺, 158.89; Ca₂PO₃⁺, 174.88; Ca₅P₂O₇⁺, 484.12; Ca₆P₂O₁₂⁺, 540.04; Ca₇P₃O₁₄⁺, 595.87; Ca₉P₃O₁₄⁺, 651.79; Ca₉P₃O₁₆⁺, 707.65). Separation of the hydroxyl ion OH⁻ from Ca₉(PO₄)₆(OH) yields the mass fragment Ca₁₀(PO₄)₅⁺, 484.12. The fragment CaF⁺, 58.96, could also be identified in the mass spectrum. The fragments CH₂N⁺, 30.03; C₂H₅N⁺, 68.05; C₃H₇N⁺, 70.07, are known residues of the amino acid proline, one of the main components of collagen type 1 [30]. C₄H₈NO⁺, 86.06, can be assigned as a hydroxyproline fragment. Figure 2e shows representative mass spectra for the reference—12 months sham—and 12 months diet groups for the Ca signal. An intense Ca signal was found for the 12 months sham group. An excerpt from a typical negative mass spectrum is depicted in figure 2f. PO₃⁻, 79.96; PO₄⁻, 62.94, were found to derive primarily from the mineralized areas, CN⁻, 26.10, from non-mineralized organic compounds in spectra obtained in the negative ion mode.

2.1.2. Chemical imaging
Using the chemical information, we were able to visualize the bone specimen morphology and its composition similar to histology, but without the need for staining. In figure 3, the microscopic view of the bone tissue (figure 3a) and the corresponding ToF-SIMS image (figure 3b) are shown. The cross section, which has been prepared to visualize bone quality in a histological investigation, shows part of an osteoporotic rat vertebra. Trabeculae (Tb), osteoid (Os), bone...
marrow, connective tissue and adipose tissue (At) can be discriminated. The ToF-SIMS image recorded in the positive ion mode, collecting all positive SIs, gives an overview using the information from the lateral distribution of the Ca⁺ signal in green, the C₄H₉N⁺ signal in red and the C₄H₈ signal in blue (figure 3b). Here, the C₄H₈ signal represents residues of the adipose tissue. These three mass signals suffice to identify unambiguously the mineralized trabecular area, the non-mineralized connective tissue as well as the cell membranes of the adipose tissue. In addition to the overview image, two smaller regions (1 and 2) have been analysed in the positive as well as in the negative ion mode. The images recorded in the positive mode show an overlay of the Ca⁺ (green) signal, the C₄H₉N⁺ (red) signal and the C₄H₈ (blue) signal (1positive and 2positive). The detail image 2positive allows a distinct determination of the trabeculae (Tb) in green and the surrounding non-mineralized border, the so-called osteoid (Os), in red. The corresponding negative ion mode images show the overlay of PO₄³⁻ signals in green (Tb), CN⁻ signals in red (Os) and signals of various long-chain fatty acid fragments such as C₁₄H₁₆O⁻ in blue. Mapping signals of long-chain fatty acid fragments identifies the cellular membrane structure of adipose tissue in the negative ion mode. However, the images recorded in the negative ion mode also allow distinct discrimination between trabeculae (green), non-mineralized areas such as osteoid (red) and adipose tissue (blue).

2.1.3. Characterization of bone
From the positive SIs, we gain information about the local distribution of minerals as well as of the organic compounds in the bone. The Ca images shown in figure 4c,g,h exhibit a quite heterogeneous distribution of Ca all over the trabecular meshwork. In addition, numerous microfractures within the trabecular bone are visible. In the immediate vicinity of some fractures, calcium is accumulated, or the fractures are located particularly in areas of high Ca content. Furthermore, the fibril structure of collagen type 1 can be recognized to some extent. The mass images of Ca⁺ and C₄H₈N⁺ provide complementary information about the distribution of hydroxyapatite and collagen, as shown in figure 4e,f.

2.2. Analysis of diseased bone
2.2.1. Scans of vertebrae
The mass images of Ca⁺ and C₄H₈N⁺ depict the structure of rat vertebrae from the 12 months sham group (figure 5a,b)
Figure 4. ToF-SIMS images (a) of an osteoporotic trabecula showing the Ca\(^{+}\) distribution (500 × 500 μm\(^2\), Bi\(^{+}\), high-current bunched mode) and the contour of the collagen structure (white). (b) Corresponding C\(_4\)H\(_8\)N\(^{+}\) image representing the collagen structure of the trabecula (a). (c) 500 × 500 μm\(^2\), Bi\(^{+}\), image of the trabecular meshwork obtained in the unbunched mode for high lateral resolution showing numerous trabecular microfractures. (d) 49.5 × 49.5 μm\(^2\) image, Bi\(^{+}\), obtained in the unbunched burst mode for the determination of the Ca content of the edge and centre regions of the trabecula. (e,f) Detailed view of (c), 99 × 99 μm\(^2\), Bi\(^{+}\), obtained in the unbunched mode for high lateral resolution showing the Ca\(^{+}\) (e) and C\(_4\)H\(_8\)N\(^{+}\) (f) distribution representing the fibril structure of collagen. (g,h) Ca\(^{+}\) images, 500 × 500 μm\(^2\), Bi\(^{+}\), showing numerous trabecular microfractures and accumulation of Ca adjacent to the fractures (white arrow) for reference group (g) and 12 months diet group (h).

Figure 5. ToF-SIMS images of the whole vertebra of (a,b) the 12 months sham group and (c,d) the 12 months diet group showing the Ca\(^{+}\) (a,c) and the C\(_4\)H\(_8\)N\(^{+}\) (b,d) distribution, Bi\(^{+}\), high-current bunched mode, stage scans 9 × 9 (a,b) and 6 × 6 mm\(^2\) (c,d).
and the 12 months diet group (figure 5c,d). The characteristic reduction of the trabecular density and structure in the affected vertebra can be seen unambiguously (figure 5c,d). The C₄H₈N⁺ mass image of this vertebra reveals a broadening of the trabecular collagen matrix and a loss of the distinct meshwork structure. By contrast, the collagen matrix of the healthy vertebra shows an intact trabecular structure which is mineralized homogeneously (figure 5a). In addition, we found Ca accumulation at the outer part of the affected vertebra (white rectangle). This accumulation was observed for osteoporotic vertebra in several cases.

2.2.2. Evaluation of the calcium content

Figure 6 shows Ca images (500 × 500 μm²) which give an overview of the trabecular meshwork of the vertebra. Comparing the images of the reference and sham groups (healthy group) with the one of the diet groups (diseased group), the reduction in the trabecular number density and thickness with time is obvious. We also found a decrease in the Ca concentration in the affected trabeculae represented by the reduced brightness of the Ca⁺ mass image. To determine the decline in the local Ca content, numerous regions of the trabecular meshwork of each sample were analysed as described before. In figure 7a, the mean values for the Ca count rates in each group at different times are shown. There is an increase in the Ca content for the sham group compared with the reference group of up to 22%. After three months, a decline in the Ca content of 48% for the diet and steroid group compared with the three months sham group is detected. The decrease amounts to 57% and 60% for the diet group compared with the respective sham group after 12 and 14 months.

A detailed analysis of the trabecular edge and centre region revealed a reduced Ca concentration at the trabecular edge for the 12 months diet group compared with the reference and 12 months sham group shown in figure 7d. The decline in the Ca concentration in the edge region compared with the centre region is 38% for the diet group.

2.3. Statistical analysis

The evaluation of the integrated intensity of the Ca signal confirms that the reduction in the Ca content for the three, 12 and 14 months diet groups compared with the reference group is statistically significant (p < 0.05). The reduction in the 12 and 14 months diet groups is of even higher significance (p < 0.001). By contrast, the decline in the Ca content after three months in the steroid group compared with the reference group is considerable but is not statistically significant. A comparison of the Ca content after three months shows a significant reduction in the Ca content for the diet group as well as for the steroid group compared with the sham group. After 12 months, there is also a significant reduction in the Ca content for the diet group compared with the corresponding sham group, as expected. In addition, the reduction in the Ca content after 14 months compared with the three months and 12 months sham groups is statistically significant. In addition, the decline in the Ca content of the 14 months diet group compared with the three months sham group is again highly significant (p < 0.001). All results are summarized in figure 7a.

2.4. Characterization of the trabeculae

In contrast to the decrease in the Ca concentration, the intensities of the C₄H₈N⁺ signals representing the collagen matrix remain constant. A slight increase according to the ongoing grade of bone deterioration may be inferred. In figure 4a, we sketch the border of the collagen signal of a diseased bone with white lines in the Ca⁺ mass image. The C₄H₈N⁺ signal represents the collagen matrix and therefore the actual area of the trabeculae (figure 4b). Ca is located only in the centre of the trabeculae. In this figure, we clearly see the extent of the degradation process. This significant decline is again accompanied by a heterogeneous distribution and numerous microfractures.

2.5. Ca/P ratio

A further parameter that may be used to assess bone quality is the variation of the Ca/P ratio within the different groups over

Figure 6. ToF-SIMS images (500 × 500 μm²; Bi⁺) obtained from cross sections of rat vertebrae. The images show the distribution and the intensity of the Ca signal across the trabecular meshwork. (Colour scale from black, to red to yellow depending on the Ca content.)
M1
M1
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(g cm
decrease in the Ca/P ratio was found for the treated groups. Using the technique can be considered as quasi-non-destructive. Using the desorbed ions, such as in an ion microscope. The amount is possible to sustain the spatial correlation and distribution of secondary ions (SIs) from the first atomic layers (up to 10 nm). Surface, which results in a collision cascade and an emission of their energy (typically 25–30 keV) is transferred to the sample logically samples. As a result of the impact of these primary ions, particularly in figure 1. The samples are bombarded with Bi3þ ions or Bi5þ clusters; the latter are most appropriate for organic and bio-
clusters; the latter are most appropriate for organic and bio-
ty (covalent bonding) of C16O4H8N+.

Figure 7. (a) Summary of the results for the Ca content of rat vertebrae for the different groups at different times. The mean values and the standard deviation of the mean value are depicted. The asterisks indicate the significance level (*p < 0.05, **p < 0.01, ***p < 0.001). (b) Results of DEXA measurements: BMD (g cm−2) of sham and diet groups over time (two-way ANOVA and Bonferroni’s multiple comparison test) and longitudinal variation with time (Friedman’s test and Dunn’s multiple comparison test) [31]. (c) Ca/P ratio for the different groups as a function of time. (d) Ca and C16H8N+ content of the edge and centre region of the trabeculae for several groups.

time. Therefore, the mean values of the Ca and P content and their ratios were calculated for each sample. The results are shown in figure 7c. In general, an increase in the Ca/P ratio with time was observed for the untreated reference and sham groups. Furthermore, at three, 12 and 14 months a considerable decrease in the Ca/P ratio was found for the treated groups compared with the reference or corresponding sham groups.

3. Material and methods

3.1. Time-of-flight secondary ion mass spectrometry

A good description of the ToF-SIMS method can be found in the literature [32–34]. ToF-SIMS originates from inorganic elemental analysis and is one of the most important and versatile surface analytical techniques. The SIMS process is illustrated schematically in figure 1. The samples are bombarded with Bi+ ions or Bi5þ clusters; the latter are most appropriate for organic and biological samples. As a result of the impact of these primary ions, their energy (typically 25–30 keV) is transferred to the sample surface, which results in a collision cascade and an emission of secondary ions (SIs) from the first atomic layers (up to 10 nm). The SIs are collected and analysed by their ToF according to their mass to charge ratio. By rastering the primary ion beam, it is possible to sustain the spatial correlation and distribution of the desorbed ions, such as in an ion microscope. The amount of ionized and removed material corresponds to a few atomic/molecular monolayers in the static mode of ToF-SIMS. Therefore, the technique can be considered as quasi-non-destructive. Using a more intensive ion gun (sputter gun), depth profiling is possible by removing layer after layer in a small defined area.

The development of cluster ion guns has enabled the analysis of biological samples and polymers in the past decade. Today, state-of-the-art SIMS analysis of organic samples is performed with bismuth or carbon clusters such as Bi5+, Bi5+, Bi5+, C60 or C60, which reduce the fragmentation and enhance the secondary ion yield of larger ion fragments.

ToF-SIMS studies on mineralized tissue revealed hydroxyapatite fragments with higher masses using a cluster ion source [25]. Owing to the high sensitivity of the method, even traces of chemical compounds are detectable, which may contain valuable information about the sample. Mass spectrometric imaging offers element-specific concentration maps of small and wide areas to trace, for instance the Ca distribution within the bone. Therefore, we present mass images of wide-area overviews (up to 9 × 9 mm2) as well as high-resolution images of regions as small as 50 × 50 μm2, with a lateral resolution of less than 300 nm.

ToF-SIMS data were acquired using a ToF-SIMS 5-100 machine (IonTOF Company, Münster, Germany) equipped with a bismuth cluster primary ion source (25 keV) and Cs+, O2+ and C60+ sputter guns. Ions were registered within the mass range from m/z = 1 to 800 u for positive and in some cases for negative SIs. Images were taken in the high-current bunched (hc-bu) mode of the primary ion gun with high mass resolution of more than 8025 full width half maximum (FWHM) at m/z = 29.00 u (e.g. the fragment CHO) and a spatial resolution of less than 10 μm. Images of high lateral resolution less than 300 nm, but with poorer mass resolution (95 FWHM at m/z = 29.00 u), were recorded in the non-bunched burst alignment (bi-ba-i) mode.
Bi primary ions were supplied with a target current of 0.2–0.5 pA for the hc-bu mode and of 0.02–0.1 pA for the bi-ba-i mode. The fields of view ranged from wide-area overviews of the whole vertebra of 9 × 9 mm² (figure 5a–f), medium-sized areas of 500 × 500 μm² (figures 3a,b, 4a–c, h,i and j) to small-area images of 99 × 99 μm² (figure 4c,f) and of 49.5 × 49.5 μm² (figure 4d,i). The small-area images of 49.5 × 49.5 μm² size were taken in the hc-bu mode to analyse the Ca concentration. The resolution was 128 × 128 pixels for small-area images as well as for the 500 × 500 μm² images (figure 6). The resolution of the 99 × 99 μm² images (figure 4c,f) and for 500 × 500 μm² image (figure 4c) was 512 × 512 pixels. The resolution of the 500 × 500 μm² images shown in figure 3a,b, taken in the bi-ba-i mode, was 1024 × 1024 pixels. All images except for those in figure 3 are presented in a colour scale ranging from black to red to bright yellow depending on the mass signal intensity. Bright colours represent high signal intensities, i.e., large SI yields. During all measurements, charge compensation was applied. All SI yields are normalized to the applied primary ion current. For data evaluation, the IonTOF software SurfaceLab v. 6.3 was used.

For a semi-quantitative comparison of the Ca concentration of affected and normal bone, we defined three to five measurement areas with a size of 49.5 × 49.5 μm² on the trabeculae of each sample and calculated the average count rates for Ca (figure 7a), normalized to the primary ion current. The error is given as the average deviation from the calculated mean value. In addition, the averaged count rates for P were also calculated to give the Ca/P ratio for all examined groups of samples (figure 7c).

A semi-quantitative analysis of the Ca concentration of the edge and centre regions of the trabeculae was carried out for the sham (12 months), reference and diet (12 months) group of animals (figure 7d). Therefore, areas of 49.5 × 49.5 μm² were analysed using Bi, bi-ba-i-burst mode, at a resolution of 256 × 256 pixels over 500 scans. Afterwards, the area was divided into four equal regions of interest (ROIs), and the Ca content of each ROI was calculated by data reconstruction. For each case, edge and centre, two ROIs and the corresponding averaged count rate of Ca were calculated and compared (figure 4d).

### 4. Discussion

ToF-SIMS as a non-routine method in medicine was applied to analyse osseous tissue and to study the ongoing grade of bone deterioration in an animal model. We analysed affected and normal bone and therefore focused on the main components of the bone, Ca and collagen.

The analysis of mineralized areas revealed typical hydroxyapatite fragments characterized by a sequence of masses separated by the mass of CaO (m/z = 55.96 Da), as recently reported by Malmöberg et al. [25]. Molecular organic fragments, such as CH₃N⁺, 30.03; C₅H₇N⁺, 68.05; C₆H₅N⁺, 70.07; and C₆H₆N⁺, 86.06, were detected and identified to be characteristic residues of glycine, proline and hydroxyproline, the main amino acids of collagen [30,36]. We consider these signals also to be characteristics for the collagen matrix of the composite material bone. This is supported by the analysis and interpretation of numerous mass images of the trabeculae. The spatially resolved detection of these fragments provides information on the localization of the collagen matrix within the bone. The good lateral resolution allows imaging of the fibril structure in high-resolution mass images (figure 4c,f). Areas of non-mineralized osteoid at the interface of trabeculae and bone marrow could be localized (figure 3). By mapping the collagen signals, we identified regions of high and low collagen content (always complementary to the Ca distribution). The complementary characteristics of the collagen matrix and the hydroxyapatite correlate well with the deposition process of hydroxyapatite along the collagen fibrils [37]. In addition, negatively charged ions such as PO₄³⁻, PO₂⁻, CN⁻ or long-chain fatty acid fragments were used to identify cellular structures within the osseous tissue. In particular, the PO₄ ion as well as the PO₂⁻ ion provide rich information: on the one hand, these ions originate from the mineralized part of the bone, represented by hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂), whereas, on the other hand, these ions are characteristic of cellular parts of the bone tissue being residues from the phospholipids of the cell membrane and also of the phosphate groups of the chromosomes in the cell nucleus.

The distribution of Ca within the bone tissue is an important parameter to assess bone quality [29,38]. It is known from the literature that heterogeneity of mineralization affects the local material properties and therefore bone quality. Contradictory studies on the distribution of minerals and the associated fracture risk in osteoporotic bone can be found in the literature [29,39,40]. We determined the spatial distribution of calcium, phosphate and several calcium phosphate ion fragments originating from hydroxyapatite. Our results confirm a heterogeneous distribution of Ca for the healthy as well as for the affected bone. However, an increased heterogeneity for the treated groups could not be determined. For the reference group, we observed the...
collagen matrix on average being covered homogeneously with Ca but with local increased concentrations especially at interfaces of microfractures. These small accumulations appear to be characteristic for young and healthy bone. The microfractures, which are scattered all over the trabeculae, are the result of mechanical stress and have already been described in the literature [41–43]. Microcracks release strain and avoid complete fracture. The occurrence of microfractures and the subsequent remodelling are consequences of the normal functional loading of the skeleton and its adaptation to individual physical demand. While an increased number of microfractures in the case of older age or osteoporosis is described for human trabeculae [39], our study on rat bones does not confirm this. We cannot show unequivocally a significant increase in microcracks for the groups with osteoporotic phenotype. This might be due to the fact that physical stress, on the one hand, and altered mechanical conditions, on the other hand (hyper-mineralization for example), have a lower impact on microspecimens (rats) than on macrospecimens (humans).

Nevertheless, the microcracks exhibit different characteristics for the reference and sham groups than for the treated groups. For the non-affected trabeculae, a Ca accumulation directly localized at the microcracks is found (figure 4g). Apparently, those microcracks arise in the areas of higher mineral content with reduced elasticity and higher brittleness. The increased Ca concentration suggests increased remodelling activity at the location of the crack. In the case of diseased trabeculae, Ca accumulations along the fractures are more expanded and randomized (figure 4h). Although the dehydration step during the preparation process also causes fractures, the exclusive appearance of microcracks as artefacts can be excluded. The characteristic accumulation of Ca and the complementary structure of the collagen matrix right at those fractures allow the distinct differentiation between microfractures and artefacts. A heightened Ca intensity as a result of edge effects can be excluded for the same reasons.

Furthermore, a remarkable decrease in the Ca content can be documented from the centre to the edge region for the diet group with time in a semi-quantitative analysis. We suggest that this is caused by the disturbed mineralization process owing to osteoporotic conditions. An unbalanced remodelling process may account for overactivity of the osteoclasts, which release hydroxyapatite and the collagen matrix. We suppose that the enzymatic dissolution of the collagen matrix is not complete, hence parts of the collagen matrix remain unchanged. In the following step of the remodelling process, osteoblasts start to rebuild new bone by expressing extracellular matrix as a scaffold for the crystallization of calcium phosphate crystals. Owing to the calcium, phosphorous and vitamin D deficiency caused by the deficit in nutrition, the mineralization process is extenuated or even impossible [44]. The result is a heterogeneous distribution of Ca, sometimes leading to a complete absence of minerals in wide regions. The extent of non-mineralized collagen might be an argument for an induced osteomalacia rather than osteoporosis. This has to be confirmed by further studies. Stage scans of the whole vertebra clearly show the extent of the pathological degradation process. The release of the minerals leads to a trabecular meshwork consisting mainly of collagen, as shown in figure 5d. Considering the whole vertebra, it becomes apparent that, in unstressed regions of the inner part, an extended Ca depletion can be found. By contrast, an accumulation of calcified tissue is found in the outer region. This part of the vertebra sustains the largest portion of physical strain. As remodelling and mineralization is adapted to stress-related strain, these burdened regions exhibit an increased content of calcium [45,46].

Ca mass images of the trabecular meshwork also showed a remarkable reduction in the trabecular number and thickness, which would be in accordance with an osteoporotic disease pattern [47]. In addition, the semi-quantitative evaluation of the Ca content revealed a significant decline in Ca for the affected groups. Here, the ToF-SIMS data show the same tendencies as the corresponding DEXA measurements, as shown in figure 7b. But, the decline of Ca (in terms of percentage) clearly exceeds the decrease in BMD obtained by DEXA measurements. This is probably due to the selectivity of ToF-SIMS, which allows the exclusive evaluation of the Ca signal, whereas DEXA integrates all X-ray absorbing components such as Si, Mg or Sr. Furthermore, the natural growth of the animals’ skeletons has to be considered as it results in increasing bone thickness and bone volume. For the DEXA data evaluation, it was not possible to account for the parameter bone thickness sufficiently. Therefore, the calculated BMD values are systematically too high with ongoing age of the rats. It has to be noted that SIMS does not a priori offer direct information on absolute concentrations. The count rates of the mass signals are proportional to the surface concentrations of the respective species, but they also depend on a number of other sample properties, which influence the ionization process. A substantial decrease in the Ca content owing to pathological modifications of the bone influences the composition of the matrix and therefore the ionization probability for Ca. So, for a semi-quantitative comparison of the Ca concentration of affected and normal bone, all samples have been prepared in the same way, and identical measurement conditions were applied to give good reproducibility. For a quantitative interpretation of the measured mass signal intensities, the matrix effect has to be considered, and calibration with suitable standards is necessary. We are currently defining such standards and are aiming for a reliable quantification of the Ca content using ToF-SIMS in a future study. Our own preliminary measurements showed a linear correlation of the Ca signal with the Ca content within the range of 40–80% Ca in a collagen matrix.

As known from the literature, the building of hydroxyapatite Ca₁₀(PO₄)₆(OH)₂ proceeds via several stages with various calcium phosphate phases as precursors [48]. Amorphous calcium phosphate as well as octa calcium phosphate are discussed as potential biologically relevant precursors of hydroxyapatite in bone [48]. The different calcium phosphate phases are characterized by their chemical formula and their Ca/P ratio [49]. The local Ca/P ratio of the bone indicates the stage of mineralization and allows the assessment of bone quality. Therefore, the analysis of the Ca/P ratio with time can provide supplementary information about the mineralization process and its alteration in systemically diseased bone. Diverging results have been published so far on this subject. While Zoehrer et al. [29] found an increased Ca/P ratio based on imbalance and decrease of P in the case of osteoporosis, the study by Basle et al. [50] showed no differences. By contrast, Tzaphlidou et al. as well as Kourkoumelis et al. reported a correlation of
osteooporosis with decreased Ca/P ratio in various studies [51–53]. We evaluated the Ca/P ratio for the different groups after defined time intervals. For the untreated and healthy groups (reference and sham), we observed an increasing Ca/P ratio with time. This is explained by the advancing age of the animals and crystallization of young to mature bone, respectively. The density of densely mineralized regions increases with age [5,6,54]. The study by Zoehrer et al. [29] reveals that areas of higher mineralization exhibit an increased Ca/P ratio, and our results are in accord with this. By contrast, the Ca/P ratio of the corresponding diet groups shows a decrease in the Ca/P ratio with time. As we did not observe regions of high calcium content and areas of low Ca content in particular, as Zoehrer et al. [29] reported for human bone, the decrease in the Ca/P ratio appears to be characteristic for the treated groups, indicating lower bone quality and therefore osteoporotic patterns.

We could confirm, in the present study, that alterations of the chemical composition of the inorganic and organic matrix have to be taken into account when estimating bone quality [16,37,53]. Our study also emphasizes the need for imaging methods, which facilitate the examination of both to elucidate disease patterns in more detail. Owing to the higher spatial resolution than FT-IR [16] (300 nm), good mass resolution (R > 8000) and high sensitivity (in the ppm range), ToF-SIMS has been found to be a valuable technique to address this task. So, the results presented herein show typical osteoporotic characteristics referred to the minerals but compelling arguments for osteomalacic patterns when considering the organic matrix. Therefore, our ToF-SIMS study revealed that the experimental set-up did not induce osteoporosis in the diet group unambiguously, but osteomalacia. Clearly, technical improvements will further advance the application of ToF-SIMS; for example, the development of standards for the quantitative analysis of bone components and a distinct mapping of the calcium phosphate phases to assess and track the mineralization process in more detail.

Using the chemical information obtained by the mass spectra, it will be possible to learn more about the mechanisms taking place in bone such as the complex bone remodelling process. The parallel detection of all chemical information deriving from the sample surface offers a wide range of promising opportunities. Messenger molecules can be detected, and special biomarkers and drugs can be traced, once their fragments are identified unequivocally and if the local concentration of these compounds is high enough. The characterization of modified implant surfaces and the analysis of the interface between the implant and bone tissue will support the development of improved materials and drugs for patients with systemically diseased bones. Nevertheless, two important and critical aspects should be noted. First, sample preparation has to be performed carefully and, second, the low concentration of biomolecules with high masses, large molar volume and low ionization probability has to be taken in consideration. We can overcome this problem if either the concentration of the species of interest is sufficiently high or we leave the static SIMS limit. In any case, ToF-SIMS facilitates the collection of valuable compositional and structural information of complex biological tissues such as bone as a complement to other techniques.

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