Time of flight secondary ion mass spectrometry of bone—Impact of sample preparation and measurement conditions

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Time of flight secondary ion mass spectrometry (ToF-SIMS) enables the simultaneous detection of organic and inorganic ions and fragments with high mass and spatial resolution. Due to recent technical developments, ToF-SIMS has been increasingly applied in the life sciences where sample preparation plays an eminent role for the quality of the analytical results. This paper focusses on sample preparation of bone tissue and its impact on ToF-SIMS analysis. The analysis of bone is important for the understanding of bone diseases and the development of replacement materials and new drugs for the cure of diseased bone. The main purpose of this paper is to find out which preparation process is best suited for ToF-SIMS analysis of bone tissue in order to obtain reliable and reproducible analytical results. The influence of the embedding process on the different components of bone is evaluated using principal component analysis. It is shown that epoxy resin as well as methacrylate based plastics (Epon and Technovit) as embedding materials do not infiltrate the mineralized tissue and that cut sections are better suited for the ToF-SIMS analysis than ground sections. In case of ground samples, a resin layer is smeared over the sample surface due to the polishing step and overlap of peaks is found. Beside some signals of fatty acids in the negative ion mode, the analysis of native, not embedded samples does not provide any advantage. The influence of bismuth bombardment and O₂ flooding on the signal intensity of organic and inorganic fragments due to the variation of the ionization probability is additionally discussed. As C₆₀ sputtering has to be applied to remove the smeared resin layer, its effect especially on the organic fragments of the bone is analyzed and described herein. © 2015 American Vacuum Society.

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I. INTRODUCTION

Osseous tissue is a complex composite material. To a great extent, it is built of extracellular matrix that mainly consists of collagen type I fibrils and mineralized hydroxyapatite crystals.¹ The cellular matrix includes various cell types like osteoblasts, bone lining cells, osteoclasts, and osteocytes. In view of this wide variety of components, the analysis of bone tissue is a nontrivial task. X-ray scanning techniques such as dual-energy x-ray absorptiometry and quantitative computed tomography are used in the clinic to evaluate the bone mineral density as an indicator for

osteoporosis, one of the most frequent forms of bone disease in age.^{2,3} In addition, biopsies of osseous tissue are often analyzed by immunohistochemical, histological, and/or histomorphological techniques to assess bone quality. These analyses rely on sophisticated and well-established embedding, sectioning, and staining techniques in combination with microscopy.

The simultaneous analysis of organic components and the inorganic mineralized part of bone tissue is particularly difficult. However, this type of analysis is important to understand the complex mechanisms of the bone remodeling process and its alteration in the course of disease. The analysis of degenerated bone or the investigation of implanted material and its interface to the bone is also quite challenging due to the large differences in the materials, but this type of characterization

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is indispensable to assess the osseointegration process. Also, the spatially resolved detection of pharmaceutical agents in bony tissue is desired, because this would provide valuable information for the optimization of the treatment of bone disease patients.

In view of these demands, time of flight secondary ion mass spectrometry (ToF-SIMS) with both its high spatial resolution as well as its good mass resolution has become an important surface analytical method, which provides detailed chemical information.⁴ SIMS imaging delivers the spatial distribution of the components with a lateral resolution down to 100 nm along with a detailed mass spectrum. ToF-SIMS can detect ions from both organic compounds like collagen as well as the minerals of the bone. New developments in ion guns have also improved the quality of depth profiles.^{5,6} The applicability of ToF-SIMS for analyzing bony tissue, implants, and for tracing of pharmaceutically active compounds in mineralized tissue has been demonstrated successfully within the last years.^{7–15}

In order to take advantage of the benefits of ToF-SIMS, the sample preparation and the measurement conditions have to be optimized to ensure reproducible, comparable, and high quality data. The critical role and the importance of an optimized sample preparation route for obtaining high quality data is well known in the field of imaging mass spectrometry. For example, sample preparation for matrix assisted laser desorption ionization-mass spectrometry analysis of soft tissue has been discussed in literature.¹⁶ For ToF-SIMS analysis, only a few studies have been published so far on the effect of sample preparation, typically dealing with the preparation of cells or soft tissue.^{17–21} To the best of our knowledge, no study on the sample preparation of bone tissue for ToF-SIMS analysis has been published so far.

Any embedding process has to ensure that interesting components are not rinsed out, remain at their original location, and that no sample damage due to chemical reactions occurs. Nonetheless, the embedding procedure will influence the sample, and it is important to evaluate this influence. It is the major aim of this paper to judge this influence and to work out the optimal sample preparation route for ToF-SIMS analysis, which also allows combining the advantages of ToF-SIMS with histological or histochemical investigations. Therefore, we focus on the analysis of bone samples from human femoral heads, which have been embedded in methacrylate based resins. Latter are typically used for histological investigations. The commercially available and routinely used Technovit 9100 resin (Heraeus Kulzer) allows comprehensive enzyme histochemical and immunohistochemical investigations while the mechanically harder methacrylate resin Technovit 7200 (Heraeus Kulzer) is rather used for standard staining such as toluidine-blue or hematoxylin-eosin. In order to combine transmission electron microscopy (TEM) and ToF-SIMS analysis, the samples should be embedded in epoxy resin, because for TEM analysis, bone is usually embedded in this resin with OsO_4 as contrast medium and cut in semithin sections with a thickness of less than $1\text{ }\mu\text{m}$.

Cut and ground sections of bone and control samples without embedding were analyzed by ToF-SIMS for comparison. As the sample surface is most important for reliable ToF-SIMS data, the roughness of the surfaces was characterized using confocal microscopy. For optimal mass and spatial resolution, the sample surface should be as plane and smooth as possible. As mass spectra of biological samples are usually rich in information, we used principal component analysis (PCA) to understand the influence of the different embedding materials on the mass spectra of both bone minerals and organic compounds. In the second part of the paper, we focus on the impact of different experimental parameters like the duration of primary ion bombardment, the effect of O₂ flooding and C₆₀ sputtering on the signal intensity of organic and inorganic fragments in the mass spectra. In Fig. 1, a schematic flow diagram of all experiments and measurements is shown.

II. EXPERIMENT

A. Samples and sample preparation

The femoral heads were obtained from patients undergoing total hip arthroplasty and were stored under sterile conditions in phosphate buffered saline (PBS solution at pH 7.2) for max. 1 day. Sections of $20 \times 20 \times 3 \text{ mm}^3$ were cut from the middle of the femoral heads with a low speed bone cutting diamond saw and stored for 3 days in 4% paraformaldehyde sodium PBS for fixation. After washing with PBS, the samples have been soaked and washed in ascending grades of ethanol. The investigations were conducted in

FIG. 1. Graphical flow diagram of the current study.

accordance with the ethic committee of the Medical Faculty of the Justus Liebig University Giessen.

The embedding materials used within this study are resins commonly used for preparing histological samples of mineralized hard tissues (undecalcified). The methacrylate based resins are commercially available by Hereaus Kulzer (Wehrheim/Ts., Germany): Technovit 7200 as well as Technovit 9100 are commonly used as embedding materials in this field.

1. Embedding with Technovit 7200

The infiltration process with Technovit 7200 was performed in six steps during 7 days. After polymerization induced by light (Exakt 520 light polymerization device, Advanced Technologies GmbH, Norderstedt, Germany) and subsequent storage for 12 h at 45 °C, ground-sections were prepared following the protocol of Donath.²² Unstained and polished thin sections of 30 μm were analyzed in the ToF-SIMS.

2. Embedding with Technovit 9100

After dehydration with ethanol, xylol 99.8% p.a. (Carl Roth, Karlsruhe, Germany) was used as intermedium for 2 days. This was followed by immersion (preinfiltration 1–3 and infiltration) for 2–4 days. Preinfiltration and infiltration were performed at 4 °C. The polymerization process took place under exclusion of air at –4 °C and lasted two days. T 9100 ground sections of 30 μm were prepared following the procedure given by Donath.²²

3. Embedding with epoxy resin (Epon)

Sections of $1 \times 2 \times 5 \text{ mm}^3$ were cut from the middle of the femoral heads with a low speed bone cutting diamond saw and fixed in 2% paraformaldehyde (Carl Roth, Karlsruhe, Germany) and 2% glutaraldehyde (25% EM Grade, Plano, Wetzlar, Germany) with 0.02% picric acid (Fa. Riedel de Haen, Seelze, Germany) in 0.1 M sodium cacodyl buffer (cacodylic acid sodium-salt trihydrate, Carl Roth, Karlsruhe, Germany) for 24 h at 4 °C. Afterward, the samples were washed with sodium cacodyl buffer solution. Some samples underwent a second fixation step using 1% osmium tetroxide solution and washing again with sodium cacodyl buffer solution. Dehydration was achieved by using ascending grades of ethanol and xylol as intermediate. The epoxy resin was composed of 48% glycid ether 100, 31% methyl nadic anhydride, 20% 2-dodecenylsuccinic acid anhydride, and 1.5% 2,4,6-tris (dimethylaminoethyl)phenol (DMP30) (all reagents from Serva, Heidelberg, Germany). Semithin sections of 0.5–1 μm thickness were cut with a histology diamond cutter (Reichert-Jung Ultracut, Fa. Leica Microsystems, Wetzlar, Germany).

4. Native bone samples

The femoral head sections were stored at –20 °C in the refrigerator and cut into 2 mm thick slices with an Isomet

low speed saw. These slices were polished with ascending grades of sand paper (4000 grit and 6000 grit) to remove the outermost layer and to obtain the smoothest possible surface without any scratches (checked with the light microscope). The slices were washed with analytical reagent grade ethanol to remove salts and residues of bone marrow and were dried and degassed for several days before measurements.

B. ToF-SIMS-measurements

All SIMS measurements were performed with a ToF-SIMS 5–100 machine (IONTOF Company, Münster, Germany). The machine is equipped with a 25 kV Bi cluster primary ion gun, 2 kV Cs^+ and O_2^+ sputter guns, and a 10 kV C_{60}^+ -gun. For data evaluation, the SURFACE LAB 6.4 software (IONTOF Company, Germany) was used.

Bi_3^+ -ions were used as primary ions, and C_{60}^+ -ions with 10 keV energy ($500 \times 500 \mu\text{m}^2$) were used for cleaning purpose. Charge compensation was achieved with a defocused low energy electron gun in all SIMS measurements. The data evaluated by PCA were obtained in the high mass resolution mode (high-current-bunched mode) using Bi_3^+ as primary ions of an area of $50 \times 50 \mu\text{m}^2$ with 128×128 pixels and 100 scans applying a primary ion dose of $<1.5 \times 10^{13}$ ions cm^{-2} to maintain almost static conditions. In the positive ion mode, the cycle time was 100 μs , and in the negative ion mode 85 μs [$I(\text{Bi}_3^+) = 0.3\text{--}0.4 \text{ pA}$]. In total, five different types of samples (shown in Fig. 1) of three different donors have been analyzed by seven spots in the positive as well as in the negative ion modes. Positive and negative ion mass spectra were calibrated using H^+ , H_2^+ , CH_3^+ , C_2H_5^+ , C_3H_3^+ , and C_7H_7^+ and C^- , C_2^- , C_3^- , C_4^- , and C_5^- ions, respectively.

The mass resolution ($m/\Delta m$) at the C_2H_5^+ ($m/z = 29 \text{ u}$) or C_2H^- ($m/z = 25 \text{ u}$) peak was better than 4000 for all measurements in the positive ion mode and better than 3000 for all measurements in the negative ion mode.

The mass images of the bone cross sections were obtained in the high mass resolution mode with higher lateral resolution (low-current-bunched mode) of an area of $500 \times 500 \mu\text{m}^2$ with 512×512 pixels, three shots per pixel, and 13 scans with a lateral resolution of $<2 \mu\text{m}$ [$I(\text{Bi}_3^+) = 0.06\text{--}0.1 \text{ pA}$].

To evaluate the influence of Bi_3^+ ion bombardment on the ionization probability, an area of $50 \times 50 \mu\text{m}^2$ with 128×128 pixels and 1200 scans (1 shot/pixel) was analyzed. To evaluate the influence of Bi_3^+ ions and C_{60}^+ ions during a noninterlaced sputter process, C_{60}^+ ions with 10 keV were applied on an area of $500 \times 500 \mu\text{m}^2$ with a field of $50 \times 50 \mu\text{m}^2$ for Bi_3^+ analysis (analysis of 1 frame, 5 s sputter, and 3 s pause). As bone is an insulator, O_2 flooding (pressure main chamber $\sim 5 \times 10^{-6} \text{ mbar}$) was applied to reduce charging effects for all measurements with negative polarity. For measurements with positive polarity, O_2 flooding is not necessary, but measurements have been done with and without O_2 flooding for comparison and to evaluate the influence of O_2 . For cleaning purpose, C_{60}^+ ions with 10 keV energy

were applied on an area of $500 \times 500 \mu\text{m}^2$ for 300 s [$I(\text{C}_{60}^+) = 0.45 \text{ pA}$].

C. Confocal microscopy

Optical images were taken using the 2D mode of a PLu neoX 3D optical profiler (Sensofar, Terrassa, Spain) equipped with a blue LED (460 nm) and using a $20\times$ objective to evaluate the roughness of the sample surface.

D. Principal component analysis

Statistical multivariate techniques (MVA) are often used to facilitate the discrimination of most characteristic spectral data and significant signals from complex data sets. PCA is the most widely used multivariate method for the evaluation and interpretation of ToF-SIMS spectra. This nonparametric method extracts the relevant information by transforming data such that they can be represented by contributions of principal components. A detailed description of PCA is given elsewhere.²³ All ToF-SIMS data within this study were analyzed with the NESAC/BIO MVA Toolbox, which is especially designed for analyzing ToF-SIMS data by PCA.²⁴ For data evaluation, we collected data of the pure embedding material and the mineralized bone from each sample, seven spots each of three different donors. Peak selection was done manually using almost all peaks. Particular attention was taken to

include all—inorganic and organic—mass signals, which typically derive from calcium phosphates and amino acids. The data were normalized to the sum of the selected peaks and scaled by SQRT-mean centering.

III. RESULTS AND DISCUSSION

A. Surface properties

As can be seen in Fig. 2 the embedded samples show much smoother surfaces than the native bone. The native bone (without embedding) exhibits a very rough surface despite a careful polishing procedure. Focusing of the ion beam on such rough samples is often difficult. Therefore, it is challenging to generate reproducible and reliable mass spectra and images from this type of samples, which is documented by the ToF-SIMS image presented in Fig. 2.

B. Distinguish fragments of embedding material and bone

Apart from calcium and calcium phosphates all other peaks found in the positive spectra of bone samples are of the type C_xH_y^+ , $\text{C}_x\text{H}_y\text{O}^+$, and $\text{C}_x\text{H}_y\text{N}^+$. These kinds of fragments can originate from the embedding material as well as from the organic compounds of the bone. To discriminate and differentiate between these signals and to specify their

FIG. 2. Comparison of cut and ground sections with a nonembedded, native bone sample. First row: light microscopic view; second row: topography determined by confocal microscopy; third row: ToF-SIMS images of the corresponding regions. Ca^+ (in green) and $\text{C}_4\text{H}_8\text{N}^+$ (in red) as overlay.

allocation is a necessary next step. Therefore, we analyzed the pure embedding materials and the not embedded bone to carve out the characteristic mass patterns supported by PCA. As can be seen in Fig. 3(a), the score plot of PC1 and PC2 separates clearly each type of embedding material: Epon-, Technovit 9100-, and Technovit 7200 sections. Along the PC2, even a differentiation in T9100 cut and ground sections is possible. The loading plots of PC1 and PC2 give information about which peaks are responsible for the separation of the samples. So, high positive loadings are positively

correlated with samples with high positive scores and negative loadings are positively correlated to samples with negative scores. The loadings refer to peaks with relatively high signal intensities in the mass spectra of each material, which means that the signal intensity of these fragments is responsible for the discrimination between the resins. Using this information and comparison with the original mass spectra allowed listing various signals for each material in Table I.

According to the constitutional structure of the embedding materials, $C_xH_yO^+$ and $C_xH_y^+$ fragments determine the

FIG. 3. (a) Scores and loading plots of PC1 and 2 from PCA of the positive ion ToF-SIMS spectra of the pure embedding materials (Technovit 7200, Technovit 9100 cut and ground sections, Epon cut sections). [(b)–(d)] Scores from PC1 and 2 from PCA of the positive ion ToF-SIMS spectra of the pure embedding materials and native, not embedded bone and associated loading plots of PC1.

TABLE I. Loadings of PC1 and PC2 from PCA of the positive ion ToF-SIMS spectra of the pure embedding materials (Technovit 7200, Technovit 9100 ground and cut sections, and Epon cut sections) [Fig. 3(a)]. Highlighted in blue: Characteristic signals of each embedding material when compared with native bone [loading plots in Figs. 3(b)–3(d)].

Loadings referring to Epon		Loadings referring to T7200		Loadings referring to T9100		Loadings referring to native bone	
<i>m/z</i>	Label	<i>m/z</i>	Label	<i>m/z</i>	Label	<i>m/z</i>	Label
22.99	Na ⁺	43.05	C ₃ H ₇ ⁺	59.01	C ₂ H ₃ O ₂ ⁺	22.99	Na ⁺
29.00	CHO	67.06	C ₅ H ₇ ⁺	59.05	C ₃ H ₇ O ⁺	30.04	CH ₄ N ⁺
31.02	C ₂ H ₅ ⁺	69.08	C ₅ H ₉ ⁺	69.04	C ₄ H ₅ O ⁺	38.96	K ⁺
43.02	C ₂ H ₃ O ⁺	71.09	C ₅ H ₁₁ ⁺	73.07	C ₄ H ₉ O ⁺	39.96	Ca ⁺
44.05	C ₂ H ₆ N ⁺	81.07	C ₆ H ₉ ⁺	75.05	C ₃ H ₇ O ₂ ⁺	40.97	CaH ⁺
55.02	C ₃ H ₃ O ⁺	93.07	C ₇ H ₉ ⁺	85.07	C ₅ H ₆ O ⁺	55.95	CaO ⁺
57.04	C ₃ H ₅ O ⁺	95.09	C ₆ H ₇ O ⁺	107.09	C ₈ H ₁₁ ⁺	56.96	CaOH ⁺
58.07	C ₃ H ₈ N ⁺	137.13	C ₁₀ H ₁₇	109.07	C ₇ H ₉ O ⁺	70.08	C ₄ H ₈ N ⁺
84.08	C ₅ H ₁₀ N ⁺			111.05	C ₆ H ₇ O ₂ ⁺	95.92	Ca ₂ O ⁺
				115.07	C ₆ H ₁₁ O ₂ ⁺	102.92	CaPO ₂ ⁺
				121.10	C ₉ H ₁₃ ⁺	111.91	Ca ₂ O ₂ H ⁺
				125.06	C ₇ H ₉ O ₂ ⁺	158.87	Ca ₂ PO ₃ ⁺
				126.06	C ₇ H ₁₀ O ₂ ⁺	174.86	Ca ₂ PO ₄ ⁺
				127.06	C ₆ H ₇ O ₃ ⁺		
				139.07	C ₈ H ₁₁ O ₂ ⁺		

mass spectra with high signal intensity and are responsible for the differentiation between the resins. C_xH_yO⁺ fragments exhibit high intensities for Epon and Technovit 9100 samples while C_xH_y⁺ fragments are associated with the mechanical harder resin T7200. C_xH_yN⁺ fragments show up as well in the mass spectra although C_xH_yN⁺ type fragments are meant to be amino acid compounds from the organic part of the bone.²⁵ But due to the addition of several chemical compounds for the polymerization process like hardener (Tetramethylaniline, for example), accelerator [trisdimethyl(aminomethyl)phenol], or stabilizers, the presence of nitrogen containing fragments also deriving from the resin must be taken into account—for Epon as well as for Technovit.

In a necessary next step, PCA was used again to select characteristic signals of each material when compared with native bone. Figures 3(b)–3(d) depict the score and loading plots of the materials and the not embedded bone samples and show the separation of each resin from the native bone.

The comparison of the resin spectra with spectra of the bone revealed several fragments being characteristic for the embedding materials (highlighted in blue Table I). These peaks can be assigned as characteristic as they are not found in such intensity in the spectra of the other embedding materials or the bony tissue.

Interestingly, these characteristic signals do not show up with a significant high intensity when analyzing the embedded trabeculae as further shown below. This allows the assumption that the embedding material infiltrates the space between the trabeculae, as expected, but not the mineralized regions themselves. PCA of an image taken from a cut section of an in Technovit 9100 embedded trabecula confirms this drawn conclusion [Fig. 4(a)]. Characteristic signals of T9100 C₂H₃O₂⁺ *m/z* = 59.01 and C₄H₅O⁺ *m/z* = 69.04 are not found on the embedded trabecula but only in the adjacent regions of the bone marrow. In any case, C₆₀⁺ sputter cleaning increases

the count rate for the Ca⁺ signal and decreases the count rate for organic bone fragments as can be seen from Figs. 4(b) and 4(c). This effect is discussed in more detail further down.

C. Comparison of embedded and not embedded bone samples

Figure 5(a) depicts the PCA scores of the first two principal components for embedded and not embedded bone samples of three different donors. The scores of all groups are rather scattered which we attribute to the characteristic heterogeneity of bone samples originating from three different donors. Nevertheless, the score plot reveals a clear separation of the embedded samples from the not embedded ones along the PC2. Considering the positive loadings of PC2 (Table II), we note that intense signals of aliphatic fragments C_xH_y⁺ are seen to correspond with the native bone. These fragments might be residues from the polishing procedure or might derive from the organic compounds of the bone. Unfortunately, their origin could not be identified unambiguously within this study.

The negative loadings of PC2 refer among others to Ca⁺ and associated fragments like Ca₂O⁺ and Ca₂O₂H⁺, which have relatively high signal intensities in the spectra of the embedded samples. Additionally, the positive loadings of PC1 correlate almost exclusively with calcium phosphate fragments, which show high signal intensities in the mass spectra of the embedded and cut samples. From this, it can be stated that the embedded trabeculae are well suited for the analysis of the bone minerals and it proves the assumption that the embedding material does not infiltrate the mineralized part of the bone. The scattered distribution of the scores of the not embedded bone samples indicates a low quality of the mass spectra as a consequence of the rough sample surface. Without embedding, it is difficult to obtain a

FIG. 4. (a) PCA from an image of a trabeculae embedded in Technovit 9100 (cut section) and associated scores of masses derived from bone (purple) or resin (green). [(b) and (c)] Overlay of mass signals of a T 9100 ground section before and after C_{60}^+ -sputtering, embedding material (T9100), and native bone before and after C_{60}^+ -sputtering.

smooth surface, which is essential for reproducible ToF-SIMS measurements.

If we concentrate on the ground sections of the embedded samples, the negative loadings of PC1 refer to several organic fragments with relatively high intensities, as shown in Fig. 5(b). Although we suppose that the embedding materials do not infiltrate the trabeculae, a small resin layer seems to be smeared over the sample surface during the polishing procedure. The relative high intensity of C_6H_9^+ at $m/z = 81.07$ supports this assumption as it refers to the resin T7200 (Table II). Otherwise, hardly any other fragment given by the negative loadings of PC1 correlates with characteristic signals of the resins listed in Table I. Hence, we conclude that the fragments derive originally from the bone.

Nevertheless, peaks of the organic part of the trabeculae might overlap with peaks of the embedding material. This overlap of signals leads to exaggerated intensities of peaks, which is shown exemplarily in Fig. 4(b) for the $\text{C}_4\text{H}_8\text{N}^+$

peak of a ground section. $\text{C}_4\text{H}_8\text{N}^+$ is a fragment of the amino acid proline and can be used to image the collagen matrix of the bone, e.g., the osteoid. Unfortunately, the signal of the proline fragment $\text{C}_4\text{H}_8\text{N}^+$ at $m/z = 70.07$ interferes with the $\text{C}_5\text{H}_{10}^+$ peak from the T9100 resin at $m/z = 70.08$. The overlap of both signals results in an increased signal, as shown in Fig. 4(b), where the main proportion of the peak can clearly be attributed to the bone (the signal of the pure embedding material shows only less intensity). A cleaning step with C_{60}^+ removes the resin layer, and the remaining signal intensity results primarily from the $\text{C}_4\text{H}_8\text{N}^+$ fragment. In addition the PCA of the image of a cut sample presented in Fig. 4(a) illustrates that the peak at $m/z = 70.07$ originates from the bony tissue and is rather the $\text{C}_4\text{H}_8\text{N}^+$ fragment of the amino acid proline than the aliphatic residue $\text{C}_5\text{H}_{10}^+$ of the embedding material.

On the other hand, the smeared resin layer of the ground section is responsible for the decreased signal intensity of

FIG. 5. (a) Scores from PC1 and PC2 from PCA of the positive ion mass spectra of the bone from not embedded (native bone) and embedded samples (Technovit 7200 and 9100 ground sections, T9100 and Epon cut sections) of three different donors and associated loadings from PC1 (b) and PC2 (c).

the inorganic calcium phosphate fragments as shown exemplarily for Ca^+ in Fig. 4(c). The signal intensity increases significantly when the resin layer is removed by C_{60}^+ sputtering and cleaning.

D. Addition of OsO_4 to Epon resin

As thin sections of Epon embedded samples are used for TEM, it is common to add OsO_4 as fixation and dense staining to enable ultrastructural analysis.²⁶ OsO_4 fixates proteins and lipids by an addition reaction to the double bonds of the

biomolecules. However, due to its oxidative effects, OsO_4 treated samples are not useful for immunohistochemistry and their usability for ToF-SIMS analysis has to be examined more in detail.

In the lower mass region ($m/z < 100$) of OsO_4 fixed trabeculae, no significant change of signals from inorganic and organic compounds was found when compared to spectra without OsO_4 . In particular, the signals of the mineralized compounds like calcium and calcium phosphates remain unchanged. Nevertheless, amino acid fragments like $\text{C}_4\text{H}_8\text{N}^+$ showed slightly increased intensities probably due

TABLE II. Significant loadings of PC 1 and PC2 from PCA of the positive ion ToF-SIMS spectra of the trabeculae of native bone samples, Technovit 7200 and Technovit 9100 ground sections, and T9100 and Epon cut sections.

Pos. loadings (PC1) referring to cut sections		Neg. loadings (PC1) referring to ground sections		Pos. loadings (PC2) referring to native bone		Neg. loadings (PC2) referring to embedded samples (cut and ground sections)	
<i>m/z</i>	Label	<i>m/z</i>	Label	<i>m/z</i>	Label	<i>m/z</i>	Label
22.99	Na^+	30.04	CH_4N^+	29.04	C_2H_5^+	18.04	NH_4^+
38.96	K^+	43.03	$\text{C}_2\text{H}_3\text{O}^+$	38.96	K^+	27.02	C_2H_3^+
39.96	Ca^+	44.06	$\text{C}_2\text{H}_6\text{N}^+$	39.02	C_3H_3^+	28.02	CH_2N^+
40.97	CaH^+	55.02	$\text{C}_3\text{H}_3\text{O}^+$	41.04	C_3H_5^+	30.04	CH_4N^+
55.95	CaO^+	59.05	$\text{C}_3\text{H}_7\text{O}^+$	43.05	C_3H_7^+	39.96	Ca^+
56.96	CaOH^+	60.06	$\text{C}_3\text{H}_8\text{O}^+$	53.04	C_4H_5^+	43.03	$\text{C}_2\text{H}_3\text{O}^+$
95.92	Ca_2O^+	68.05	$\text{C}_4\text{H}_6\text{N}^+$	55.05	C_4H_7^+	43.03	CH_3N_2^+
102.92	CaPO_2^+	70.07	$\text{C}_4\text{H}_8\text{N}^+/\text{C}_5\text{H}_{10}^+$	57.07	C_3H_9^+	44.06	$\text{C}_2\text{H}_6\text{N}^+$
111.91	$\text{Ca}_2\text{O}_2\text{H}^+$	81.07	C_6H_9^+	65.04	C_5H_5^+	58.07	$\text{C}_3\text{H}_8\text{N}^+$
132.90	CHCa_3^+	86.06	$\text{C}_4\text{H}_8\text{NO}^+$	67.06	C_5H_7^+	59.05	$\text{C}_3\text{H}_7\text{O}^+$
158.87	Ca_2PO_3^+			69.07	C_5H_9^+	70.07	$\text{C}_4\text{H}_8\text{N}^+/\text{C}_5\text{H}_{10}^+$
174.86	Ca_2PO_4^+			77.04	C_6H_5^+	95.92	Ca_2O^+
				79.05	C_6H_7^+	111.91	$\text{Ca}_2\text{O}_2\text{H}^+$
				91.05	C_7H_7^+	132.90	CHCa_3^+

to the oxidizing and cross linking properties of OsO_4 . Osmium itself could not be found within the spectra. The influence of OsO_4 on the analysis of lipids was not investigated as lipids have been washed out during the embedding procedures described herein.

To conclude, the addition of OsO_4 does not change the chemical character of the sample, but it has an influence on the signal intensity of the organic compounds of the bone, which needs to be considered. If the focus of a study is on the mineralized part of the bone, the addition of OsO_4 has almost no impact. Refer online resource 1 in the supplementary material.²⁷

E. Cleaning the samples with C_{60}^+

To get rid of the layer of embedding material which has been smeared over the sample surface, a cleaning step using C_{60}^+ prior to analysis appears to be useful. Therefore, the influence of C_{60}^+ sputtering on the embedded samples and especially on the interesting signals like calcium or the organic fragment $\text{C}_4\text{H}_8\text{N}^+$ was investigated.

Figure 6 shows the course of the Ca^+ and $\text{C}_4\text{H}_8\text{N}^+$ signals under Bi_3^+ bombardment and C_{60} -sputtering for one sample prepared as Epon cut section, T7200 ground section, and T9100 cut and ground section.

After few seconds of C_{60} -sputtering, constant and slightly enhanced Ca^+ signals with almost comparable intensities are obtained for all samples except for Epon [Fig. 6(c)]. This is an important observation as we have found a continuous increase of the calcium and calcium phosphate signals for all samples when being analyzed or bombarded with Bi_3^+ [shown in Fig. 6(b)]. In addition, the Ca^+ signal intensities under bismuth bombardment vary strongly for the different embedded samples within the first 200 scans, which is a serious problem for comparable and reproducible analysis. This might indicate some kind of matrix effect certainly influenced by the embedding material, resulting in a variation of the ionization probability. The same observation applies for the course of the organic fragment $\text{C}_4\text{H}_8\text{N}^+$, which derives from the amino acid proline and can be attributed to the collagen type I of the bone. The intensity of the signal decreases gradually with the duration of the bombardment [Fig. 6(e)]. Interestingly, the course and decrease of the signal intensity for the ground sample differ significantly from the cut ones and show a slighter decrease. Due to the smeared resin layer, an overlap of signals from bone and embedding material is observed, which results in increased signal intensities for the organic fragments [Fig. 6(e)]. The more slightly decrease of the $\text{C}_4\text{H}_8\text{N}^+$ signal is also attributed to the resin layer that might function here as a protection layer. Under C_{60}^+ -bombardment, the $\text{C}_4\text{H}_8\text{N}^+$ signal intensity of all samples shows a big drop and a strong decline after only few sputter seconds, although C_{60}^+ is meant to be quite gentle and well suited for sputtering organic samples.⁶ Again, a slighter decrease for the ground samples is observed due to the resin layer [Fig. 6(f)].

The course of the $\text{C}_4\text{H}_8\text{N}^+$ and Ca^+ signals for the ground sections under Bi_3^+ - and C_{60}^+ -bombardment allows the conclusion that T7200 samples are covered with a thinner resin

layer as T9100 ground samples due to the mechanical hardness of the material (T7200). The course of the characteristic signals of the resins $\text{C}_4\text{H}_5\text{O}^+$ (T9100) and C_6H_9^+ (T7200) confirms this assumption as shown in Figs. 6(g) and 6(h). Again, a strong decline is observed under C_{60}^+ -bombardment.

It should further be noted that the flooding of the sample surface with O_2 influences the ionization probability of the inorganic as well of the organic fragments. For comparison, the courses of these signals with and without O_2 flooding are shown in Figs. 6(a), 6(b), 6(d), and 6(e).

Studying the depth profiles the special character of the Epon embedded samples becomes apparent which might be due to their low thickness of less than $1\ \mu\text{m}$. For these samples, we never observed a constant Ca^+ signal during Bi_3^+ or C_{60}^+ sputtering. The reduction of the organic compounds of the bone appears to influence the ionization probability of calcium to such an extent that no constant calcium signal can be detected. After 1000 sputter seconds with C_{60}^+ , the complete sample is removed [$10\ \text{keV}\ \text{C}_{60}^+(I = 0.3\ \text{nA})$].

Analyzing inorganic compounds of the bone a cleaning step using C_{60}^+ should be applied to the ground samples in order to remove the plastic layer and to obtain reliable and reproducible results. In case of the cut samples and when interested in organic compounds of the bone sputtering with C_{60}^+ might not be beneficial.

In order to evaluate this question more in detail, all embedded samples as well as native bone have been sputtered for 300 s prior analysis. The obtained spectra have been compared with spectra of nonsputtered samples again supported by PCA. The results for one sample set are presented in Fig. 7. Along PC1 and PC2, the sputtered samples can be separated from the nonsputtered samples as well as from the native bone samples.

All sputtered samples show an increased signal intensity of calcium and its phosphate fragments due to the removed organic compounds and contaminations like the aliphatic fragments of the native bone or the resin layer covering the surface (Table III).

Considering the very scattered distribution of the scores for the ground T9100 sections, a more homogeneous distribution after the sputter process is observed due to the removed resin layer. In contrast, C_{60}^+ -sputtering of the cut sections leads to a more heterogeneous distribution of the scores. Here, the C_{60}^+ -bombardment affects the organic compounds of the bone more than in case of the ground sections where the resin layer initially acts as a quasiprotection layer. For the native bone samples—although a higher intensity for the calcium phosphate fragments can be observed—the more scattered distribution of the scores after sputtering might be also attributed to the reduction of the organic compounds of the bone.

F. Negative ion mode

Analyzing bone samples in the negative ion mode provides only few characteristic and meaningful fragments of

FIG. 6. Course of the Ca^+ [(a)–(c)] and $\text{C}_4\text{H}_8\text{N}^+$ [(d)–(f)] signals under Bi_3^+ bombardment and C_{60} -sputtering for T7200 and T9100 ground sections and T9100 Epon cut sections measured on the trabeculae with dioxygen flooding [(b),(c),(e),(f)]. (a) and (d) The course of Ca^+ and $\text{C}_4\text{H}_8\text{N}^+$ signals without dioxygen flooding. (g) and (h) The course of the characteristic signals of T7200 and T9100 ground sections, C_6H_9^+ , and $\text{C}_4\text{H}_5\text{O}^+$, under Bi_3^+ bombardment and C_{60} -sputtering.

the bone tissue. Besides the PO_2^- and PO_3^- fragments from the calcium phosphates, high signal intensities of CN^- and CNO^- can be detected, which are very unspecific and derive from almost all organic compounds of the resin and the bone. The same applies for CHN^- type signals. In consequence, there is an overlap of signals from the embedding material with those from the bone tissue,

which explains the increased intensity of the peaks of the ground sections compared with the peaks of the cut sections.

Fragments of the type $\text{C}_x\text{H}_y\text{N}^-$ or $\text{C}_x\text{H}_y\text{NO}^-$ have only low intensities and cannot be assigned clearly as fragments of the bone or resin in case of the ground samples. So far, only $\text{C}_2\text{H}_2\text{N}^-$ appears to be a specific fragment of the

FIG. 7. (a) Scores from PC1 and PC2 from PCA of the positive ion ToF-SIMS spectra of trabeculae prepared as Technovit 7200 and Technovit 9100 ground sections, T9100 and Epon cut sections, and native bone sample before and after having been sputtered for 300 s. (b) Associated loading plot.

amino acid proline originating from the collagen of the bone [Fig. 8(a)].

To complete the study, also the negative ion mode mass spectra of the embedded and native bone samples have been analyzed with PCA. The score and loading plots can be seen in the online resource 2 of the supplementary material.²⁷ The loading plot of PC1 confirms the results that were obtained for the positive mass spectra. For the cut samples, we observe a quite close distribution of the scores and high signal intensities of the PO₂⁻ and PO₃⁻ signals from the calcium phosphates. This is owed to the clean surface prepared by the cutting process. On the other hand, various organic compounds containing oxygen or nitrogen have been detected with relative high intensity for the ground sections. Comparison with the

spectra of the native bone and the pure embedding material showed these signals originate from overlap of signals from the embedding material and the bone. The signals at $m/z = 79.95$ and 96.95 could be identified as SO₃⁻ and HSO₄⁻ fragments originating from additives of the polymerization process. These signals likely overlap the HPO₃⁻ and H₂PO₄⁻ fragments from the calcium phosphate of the bone. But the achieved mass resolution for the measurements in the negative ion mode is not sufficient to resolve the peaks. However, the differentiation between the embedded and not embedded samples is achieved by long chain fragments from lipids of the native bone, which have remained in the samples although the bone was washed once in ethanol. On the surface of the native bone samples, a homogeneous distribution of lipid fragments is observed. In the case of the embedded samples only residues of adipose cells in the bone marrow can be detected with some luck, but no lipids are found on the surface of the mineralized tissue anymore.

During the sputter process with C₆₀⁺, also an initial increase of the course of the phosphate signals is observed. This goes along with the course of the Ca⁺ signals in the positive ion mode. In the case of the Technovit 9100 ground sample, the signal intensities rise slower due to the smeared layer of embedding material that has to be removed first. The bombardment of the samples with Bi₃⁺ is set in a steadily increase of the signals as the Bi₃⁺ impact influences the ionization probability of the calcium phosphates in the positive as well in the negative ion mode (see online-resource 2 in the supplementary material²⁷).

Having a closer look on the signal intensities of the organic compounds during C₆₀⁺ sputtering, it becomes apparent that they all decrease remarkably already after short sputter time, as can be seen in Fig. 8. This is accompanied by a loss of mass resolution and a broadening of the peaks, which do not allow an identification of certain organic mass

TABLE III. Loadings of PC1 and PC2 from PCA of the positive ion ToF-SIMS spectra of trabecula prepared as Technovit 7200 and Technovit 9100 ground sections; T9100 and Epon cut sections; and native bone samples before and after having been sputtered for 300 s.

Pos. loadings (PC1) referring to sputtered samples		Neg. loadings (PC1) referring to not sputtered samples	
m/z	Label	m/z	Label
39.96	Ca ⁺	27.02	C ₂ H ₃ ⁺
55.95	CaO ⁺	28.02	CH ₂ N ⁺
56.96	CaOH ⁺	29.04	C ₂ H ₅ ⁺
58.96	CaF ⁺	30.04	CH ₄ N ⁺
95.92	Ca ₂ O ⁺	41.04	C ₃ H ₅ ⁺
111.91	Ca ₂ O ₂ H ⁺	43.03	C ₂ H ₃ O ⁺
158.88	Ca ₂ PO ₃ ⁺	44.06	C ₂ H ₆ N ⁺
		55.02	C ₃ H ₃ O ⁺
		56.06	C ₃ H ₆ N ⁺
		58.07	C ₃ H ₈ N ⁺
		59.05	C ₃ H ₇ O ⁺
		68.06	C ₄ H ₆ N ⁺

Fig. 8. Overlay of negative mass spectra taken from Epon resin and trabecula embedded in Epon resin (a) and native bone (b) before and after having been sputtered with C_{60}^+ for 300 s.

signals anymore. In the case of native bone, also the long chain fragments of the fatty acids are removed during the C_{60}^+ sputtering within 300 sputter seconds completely [Fig. 8(b)].

IV. CONCLUSIONS

The most appropriate embedding methods for the sample preparation of bony tissue are evaluated to ensure reliable and reproducible mass spectra but also to offer the opportunity to combine imaging mass spectrometry with histological or histochemical investigations.

The results of the present work demonstrate that the embedding materials do not infiltrate the mineralized part of the bone and that embedded samples are best suited for ToF-SIMS analysis of the inorganic compounds of the bone. The data showed that cut samples meet the requirements for reproducible measurements best due to the clean and plane/smooth surface, which offers most suitable measurement conditions and allows also the analysis of organic compounds of the bone. The usual addition of OsO_4 to Epon has no influence on the mass spectrometric analysis of the inorganic compounds of the bone, but an increase of signal intensity for the organic fragments is observed in the lower mass region.

However, during the preparation of the ground samples, a resin layer is smeared over the sample surface, which makes the analysis of the organic compounds of the bone and the distinction from the embedding material more difficult. An overlap of peaks deriving from the bone and resin is observed and increased signal intensities are obtained for several organic fragments. We could show that a cleaning step using C_{60}^+ for about 300 s is useful to get rid of the resin layer but it needs to be applied carefully, because C_{60}^+ also destroys the organic compounds of the bone. Due to this, ground sections are only recommended restrictedly for the analysis of organic compounds of the bone.

Although native bone might offer the feasibility to analyze the organic compounds without the influence of any embedding material, it has to be stated that aliphatic hydrocarbons covered the surface and constrained the analysis. Unfortunately, cleaning the surface with C_{60}^+ destroyed also the organic compounds of the bone. In addition, we could show that not embedded bone with its rough surface is not suitable for reproducible ToF-SIMS analysis. So, the use of not embedded samples is not preferable, except for the analysis of fatty acids localized on the mineralized tissue. But, in this case, it is compulsory to optimize the sample preparation for the not embedded bone or to look for another embedding method, which preserves the fatty acids.

In general, the sample preparation and measurement conditions have to be considered with great care. Samples used within one study need to be prepared in the same way and to be analyzed under the same conditions; as otherwise erroneous conclusions may be drawn. As the measurement conditions like dioxygen flooding, cleaning with C_{60}^+ , or even the bombardment with Bi_3^+ have an influence on the course of the signal intensities in the mass spectra, the chosen measurement protocol has to be kept strictly unchanged for all analyses within one study to obtain high quality, comparable, and reproducible results.

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- ²⁷See supplementary material at <http://dx.doi.org/10.1116/1.4928211> for mass spectra from cut sections of bone embedded in epon with added OsO₄ (online resource 1) and the negative ion mode mass spectra of the embedded and native bone samples analyzed with PCA as well as the course of the signals under Bi₃⁺ and C₆₀⁺ bombardement (online resource 2).