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Micro-PIXE and histochemical studies of Zn and Ca distribution in normal bone

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Abstract

To better understand the role of zinc in bone mineralization we studied the distribution of Ca and Zn by microbeam particle-induced X-ray emission (μ-PIXE) profiling and mapping, and of Zn by histochemical mapping and profiling in cortical bovine bone. For μ-PIXE, measurements were carried out at the Rossendorf nuclear microprobe with a 3.1 MeV proton beam focused to a spot of ~3 μm, at ~4–8 μm effective resolution. Maps, unique scans and side-to-side scan sequences were done for Ca and Zn. The noise in Zn profiles was filtered by Fast Fourier Transform (FFT). For histochemistry, Zn was stained in thick sections by the sulphide-silver reaction. Both μ-PIXE and histochemistry showed that Zn was localized mainly at the surfaces of various structures in bone tissue.

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1. Introduction

To study the relation between Zn and Ca in bone tissue by a topographic approach, analytical microscopy methods based on X-ray spectrometry are well suited. We previously used synchrotron radiation-induced X-ray emission (SRIXE) [1]. However, in our microbeam SRIXE measurements the spatial resolution was limited to only ~20 μm, a limitation occurring frequently with this technique due to difficulties in focusing the X-ray beam. PIXE is a similar method which excites the target by means of a proton or heavier ions beam [2] and yields significant insight in bones and other biomineral specimens [3]. μ-PIXE, which uses a focused proton microbeam, currently achieves a spatial resolution in the 1–10 μm range and, due to its multielemental character, allows also the direct comparison of Ca and Zn from the same analyzed bone areas. Moreover, μ-PIXE achieves a better sensitivity than related techniques like electron probe microanalysis (EPMA).

In this article, aimed to a better understanding of the role of zinc in bone mineralization, we present the results of our studies on the distribution and topographical relationships of Ca and Zn in bones by μ-PIXE profiling and mapping, as well as on the Zn topography by histochemical mapping.

2. Materials and methods

2.1. Materials and preparation of samples

The compact cortical bone of posterior diaphysis from metacarpal bovine bone (1 year old) was studied, and

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slices, 5–7 mm wide and 1–2 mm thick, were cut transversally and longitudinally. Bone slices were embedded in polymethyl-methacrylate (PMM) for histochemistry studies. For μ-PIXE, the slices were ethanol fixed, dried at 55 °C and diamond polished, thus providing the 'infinitely thick' targets with a flat smooth surface.

2.2. Zinc histochemistry

PMM blocks were cut using a linear precision saw (Isomet 4000) equipped with a LC diamond blade as sections 100 μm in thickness, and further thinned down to 40 μm thick by diamond polishing using a Phoenix Beta polisher. Zinc was stained in these sections by the sulphide-silver reaction (Timm's method). Stained sections were observed under optical microscopy, and images were captured with a DMX 1200F Nikon digital camera. As silver ions are deposited as metallic silver around the Zn-sulphide germs, density of silver deposition was analyzed in digitized images as gray plot profiles using the Image J (NIH) program.

2.3. Micro-PIXE measurements

The μ-PIXE measurements were carried out at the Rossendorf nuclear microprobe with a 3.1 MeV proton beam from a Tandetron accelerator [4]. An Ortec Si(Li) detector with optimized response was used. The beam had an intensity of 0.58 nA and was focused to a ~3 μm spot normally to the target, while the detector was positioned at 60°. A 270 μm thick Mylar additional absorber was used both for profiles and maps. For charge measurement, the isolated target was connected to a current integrator, the input of which was biased by a positive voltage for suppressing secondary electrons. The collected charge per profile scan was in the range of 460–580 nC. Maps $\sim 250 \times 250\ \mu m^2$ (64×64 pixels), single linear scans of $\sim 474 \times 11\ \mu m^2$ and of $\sim 948 \times 22\ \mu m^2$ (128×3 pixels) and side-to-side sequences of linear scans were done and the relative intensities for Ca and Zn were monitored. The effective size of a pixel was of 7.41 μm for most profile scans, of 3.70 μm for some profile scans, and of 3.91 μm for the maps. Hydroxyapatite was used as a reference material. Concentrations were determined from PIXE spectra by GUPIX calculations, using the appropriate options for thick target analysis. The data in the Ca and Zn profiles were displayed in relative intensities (count numbers), which was sufficient for the purpose of our study.

2.4. Fast Fourier Transform (FFT) noise filtering of the Zn profiles

As the width of each bone sample was usually up to ~6 mm, 7–8 successive profiles at ~8 μm resolution had to be performed in a single day to keep the conditions unchanged. This limited the acquisition time per scan and, therefore, in the Zn side-to-side profiles, the signal-to-noise ratio, although better than 2.0, was generally poor. Thus we had to improve the Zn profiles by smoothing out as much as possible of the noise, especially at high spatial frequencies. This could be done by Fourier transform noise filtering, which enables a controlled cutting of high frequencies. Given the discrete nature of the data, this has been done by FFT, an approximate procedure [5], which can be easily performed by a subroutine of the Origin 6.0 package.

3. Results and discussion

The Ca and Zn concentrations evaluated by μ-PIXE in the side-to-side profiles varied from a scan to another and in the mean were estimated to $17.5 \pm 4.6\%$ and 65 ± 21 mg/kg (w/w), respectively; in the maps the Ca level amounted to 15–19% and that of Zn to 73–78 mg/kg. The best detection limit for Zn in the bone samples was of 2.4 mg/kg.

Fig. 1 shows a typical Zn stained section and their corresponding plot profile. Mainly Zn is stained at the bone surfaces. Periosteal surface layer is about 50 μm thick. Endosteal layer is more unevenly, but in general it is up to 200 μm thick. Osteonic bone in the central zone is stained in single osteons at the 5–20 μm thick internal layer surrounding Havers' canal, and in cement lines.

In Fig. 2, the μ-PIXE maps of Ca and Zn in the central core of a bone as appearing in a longitudinal bone section are shown, and the corresponding intensity profiles from these maps are displayed. The three distinct maxima in the Zn profile correspond to different features in the Ca profile, i.e. to the high-slope left wing of a Ca minimum, to an element's peak at its maximum, and to no definite feature in the calcium profile. Thus in the bone section the profiles, even more visibly than the maps, point to the heterogeneous nature of the Zn peaks in correspondence with the Ca peaks. These results are similar to those provided by SRIXE; and also, they are consistent with the three distinct zinc pools in the bone as revealed histochemically and immunochemically in bone samples subjected to partial Zn extraction [1].

In Fig. 3 the panoramic, side-to-side profiles of Ca and Zn in a longitudinal section cut from the periosteum surface through the central core and to the endosteum surface are presented. Comparison of μ-PIXE side-to-side Ca profiles and FFT-filtered Zn profiles evidenced significant Zn changes associated to all Ca variations, either small or big. As the line is crossing haphazardly zones of compact mineralized tissue as well as zones of void-porous non-mineralized tissue (chiefly osteoinc canals), bone surfaces were identified as the regions where the Ca profile abruptly decayed, and here the Zn peaks were higher with respect to those in compact regions. In addition, there are also Zn peaks which apparently do not correspond to significant features in the Ca profile, resulting in a higher peak density for Zn as compared to the density of maxima-plus-minima for Ca.

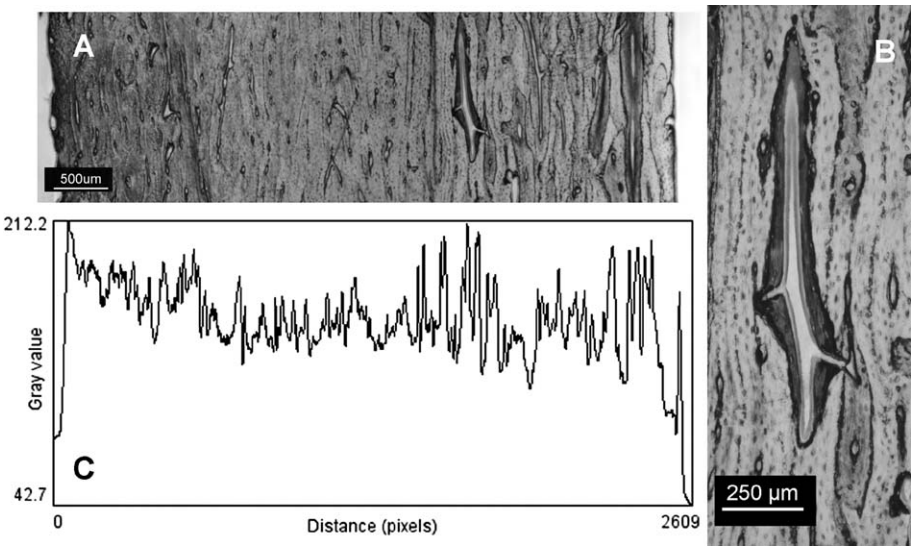

Fig. 1. (A) Panoramic view of Zn histochemistry in a longitudinal section covering all the anatomic layers of the cortical bone (periosteum, central core, and endosteum, from left to right). (B) Detail of an osteon in the central core. (C) Plot profile of silver deposition to analyze zinc distribution in (A) (pixel size, 2.16 µm).

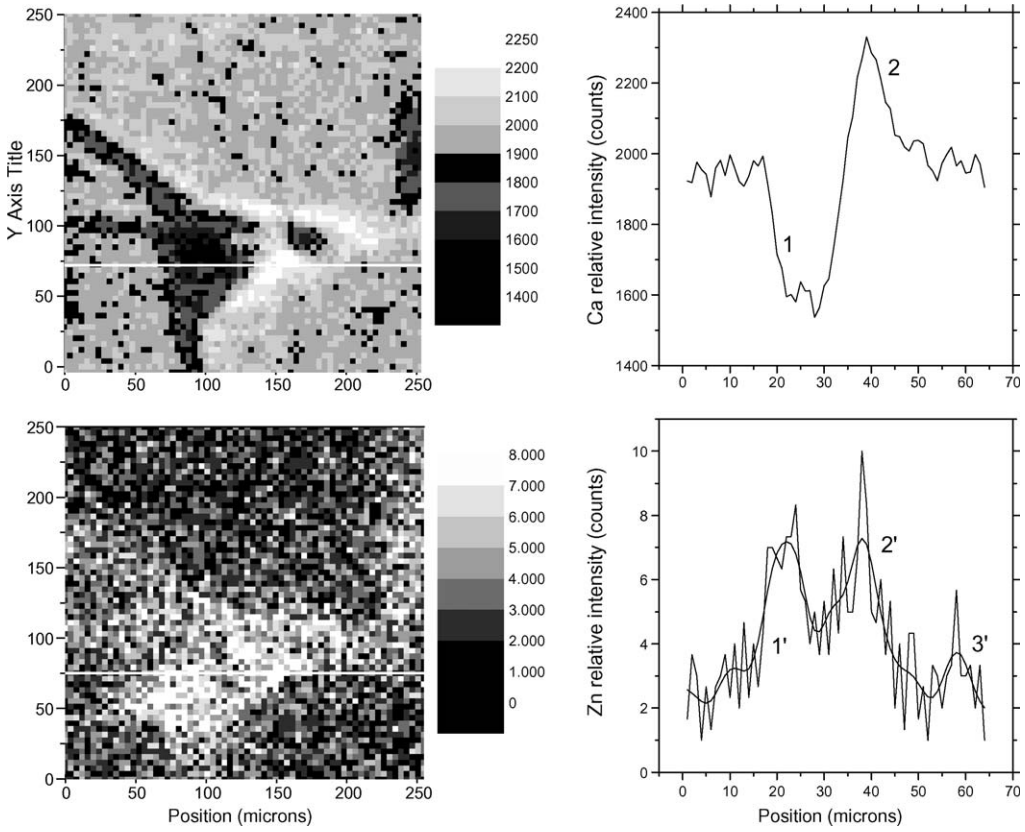

Fig. 2. µ-PIXE Ca and Zn maps and profiles of a longitudinal section in the core of the compact bone tissue. In the Zn profile the FFT-filtered data are superimposed on the initial data to evidence the reduction of noise. The Ca profile shows a minimum and a maximum, while in the Zn profile three maxima are observed. Maximum 1′ of Zn corresponds to a high slope part of Ca minimum 1; maxima 2′ and 2 of Zn and Ca have the same position; and maximum 3′ of Zn does not have any corresponding feature in the Ca profile. Therefore the Zn lateral distribution appears heterogeneous with respect to Ca. The white line shows the profile position. Pixel size (step width): 3.91 µm.

On the whole, we conclude that the results provided by the two methods – µ-PIXE and histochemistry of Zn – are showing the same trends. And, while the present data are entirely consistent with the results obtained by SRIXE [1], µ-PIXE offers a substantially better spatial resolution.

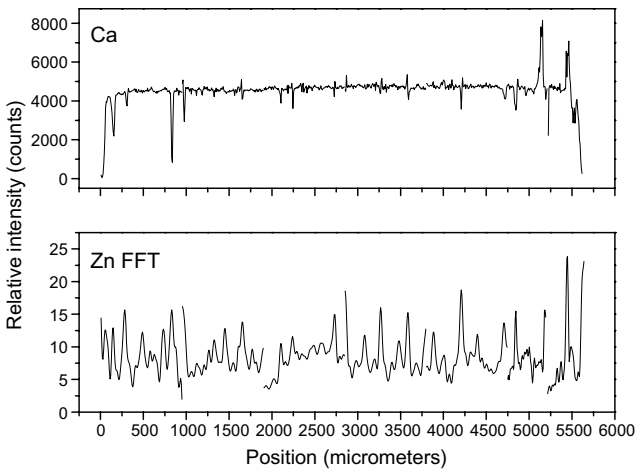

Fig. 3. Side-to-side μ-PIXE profile distributions of Ca and Zn in a longitudinal section covering all the three anatomical layers of the bone (periosteum, central core and endosteum, from left to right). The profiles were traced in seven successive scans following a direction normal to the bone specimen edges. For Zn, the FFT-filtered data are shown. Pixel size (step width): 7.41 μm.

4. Conclusions

In this study of the Ca and Zn distribution in the normal bone, one important conclusion is that both μ-PIXE and histochemistry showed Zn to be localized mainly at the surfaces of various structures in the bone tissue. Besides that, μ-PIXE maps also indicated the existence of other zinc distributions in compact mineralized zones. Moreover, the side-to-side profiling of Ca and Zn coupled to the FFT filtering of the low signal-to-noise data of Zn proved to be a valuable procedure.

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