Wieland, D.C.F.; Krywka, C.; Mick, E.; Willumeit-Roemer, R.; Bader, R.; Kluess, D.:

Investigation of the inverse piezoelectric effect of trabecular bone on a micrometer lengthscale using synchrotron radiation

In: Acta Biomaterialia (2015) Elsevier

DOI: 10.1016/j.actbio.2015.07.021

Investigation of the inverse piezoelectric effect of trabecular bone on a micrometer lengthscale using synchrotron radiation

D.C.F. Wieland[1], C. Krywka[1], E. Mick[2], R. Willumeit-Römer[1], R. Bader[2], D. Kluess[2]

[1]Helmholtz-Zentrum Geesthacht, Institut für Werkstoffforschung, Geesthacht, Germany

[2]Department of Orthopaedics, University Medicine Rostock, Rostock, Germany

Corresponding author:

Dr. Florian Wieland

Helmholtz-Zentrum Geesthacht (Außenstelle DESY)

Notkestrasse 85

D-22607 Hamburg

Germany

Phone: +49 (0)40 8998 6916

Email: Florian.Wieland@hzg.de

Abstract

In the preset paper we have investigated the impact of electro stimulation on microstructural parameters of the major constituents of bone, hydroxyapatite and collagen. Therapeutic approaches exhibit an improved healing rate under electric fields. However, the underlying mechanism is not fully understood so far. In this context one possible effect which could be responsible is the inverse piezo electric effect at bone structures. Therefore, we have carried out scanning X-ray microdiffraction experiments, i.e. we recorded X-ray diffraction data with micrometer resolution using synchrotron radiation from trabecular bone samples in order to investigate how the bone matrix reacts to an applied electric field. Different samples were investigated, where the orientation of the collagen matrix differed with respect to the applied electric field. Our experiments aimed to determine whether the inverse piezo electric effect could have a significant impact on the improved bone regeneration owing to electrostimulative therapy. Our data suggest that strain is in fact induced in bone by the collagen matrix via the inverse piezo electric effect which occurs in the presence of an adequately oriented electric field. The magnitude of the underlying strain is in a range where bone cells are able to detect it.

1. Introduction

Bone defects are critical when the ability of the bone to regenerate itself is disturbed or limited. Clinical diagnoses of bone defects with the necessity to promote bone regeneration are e.g. avascular necrosis of the femoral head, delayed fracture healing or bone tumors.

An effective adjuvant for enhanced bone regeneration is electrical stimulation therapy. Thereby, three different ways to induce an electric stimulation in bone are applied; by direct current (DC), conductive coupling (CC) and inductive coupling (IC)[1-3] DC is an invasive technology that requires the placement of electrodes directly to the bone. The electrodes are placed under the skin and connected to the power supply. CC is a non-invasive technology that uses extracorporeal electrodes on the skin above the bone lesion. IC is often referred to pulsed electromagnetic fields (PEMF), which induce electric fields in the bone lesion through a magnetic field created by an extracorporeal coil.

Kraus and Lechner designed an inductively coupled system which works at low frequencies of 12 s^{-1} and 20 s^{-1}[4]. This system is based on the interplay of two coils and two electrodes. A primary coil is placed outside of the human body while the secondary coil is an implanted transducer coil. The latter receives an induced voltage from an external alternating magnetic field and connects to two electrodes of an implant. One electrode is placed within the bone to be healed, and another is placed in the immediate proximity. Based on this approach, Mittelmeier et al. [5]proposed a bipolar induction screw system, which is clinically applied as the so-called Asnis III s-series screw (Stryker Trauma, Kiel, Germany). In this system, the transducer coil is embedded in the screw, and thus there are no extra wires to be implanted. After implantation, the patient is asked to wear an extracorporeal coil around the body three times a day for 45 min. Thereby, an external sinusoidal oscillating magnetic field induces voltage in the embedded secondary coil. In this activated area around the implant electrodes, the electric field can stimulate bone regeneration [6].

In vitro studies showed that human osteoblasts cultured under electromagnetic stimulation exhibit higher proliferation, calcium deposition, and greater expression of decorin, osteocalcin, osteopontin,

type I collagen, and type III collagen[7, 8]. On the other hand, an in vitro study using rat osteoblasts under sinusoidal magnetic fields with different intensities between 0.9 mT and 4.8 mT indicated that osteoblast proliferation was inhibited while differentiation and mineralization potentials were significantly promoted[9]. Our own research towards different parameters in electrostimulation using the bipolar induction screw system in combination with cell culture is ongoing [10]. In clinical practice, the bipolar induction screw system shows promising results [11], and exact placement of the implant needs to be taken care of due to the its influence on the electric field distribution[12]. An extensive literature review into clinical studies of electrical stimulation indicates an enhancement of bone healing [13]; however, with regard to levels of evidence of the studies and limited comparability of the different methodologies an optimization of electrical stimulation for clinical practice could not be drawn. The authors conclude that the exact mechanism by which electrostimulation enhances bone regeneration is still not fully understood and needs more investigation. It is assumed that a reciprocal piezoelectric effect in combination with piezoelectric properties of bone tissue [14, 15] is the underlying mechanism which in turn results in mechanical stimulation of bone. Nevertheless, the response of the bone to the electromagnetic field is unclear and adjustment of stimulation parameters has yet been performed only empirically. The question remains which is the optimal electrostimulation method in combination with which electric parameters. Therefore, a deeper insight into the inverse piezoelectric behavior of bone is required.

To this end, we have investigated the effect of electro stimulation on femoral bone by scanning X-ray diffraction (XRD) experiments to investigate changes in the crystal structure of hydroxyapatite (HA) with respect to the orientation to electric field. In these experiments we simultaneously recorded small and wide angle scattering data from the HA crystals. By this we were able to determine the local orientation of the HA crystals and their lattice constant with high spatial resolution. The evaluation of both scattering signals was necessary as the textured two dimensional WAXS pattern was not completely recorded due to experimental constrains. To our knowledge, the presented study

is the first to investigate the inverse piezoelectric behaviour with a spatial resolution across the bone on a micrometer length scale using scanning X-ray diffraction.

2. Materials & Methods

The scanning X-ray diffraction and scattering data with in situ application of an electrostatic field were recorded at the Nanofocus Endstation of P03 beamline [16, 17] of PETRA III synchrotron radiation source (DESY, Hamburg, Germany) and at beamline X9 [18] of the NSLS synchrotron radiation source (Brookhaven National Laboratory, Upton NY, USA). The experiments were performed in transmission mode and data were recorded in both, wide and small angle regimes (WAXS & SAXS) using a monochromatic beam with a photon energy of 13.0 keV (P03) and 13.5 keV (X9), i.e. at wavelengths of 0.954 Å and 0.918 Å, respectively. The beam sizes (horizontal × vertical) at the sample position were 1.5 × 1.5 µm² (P03) and 20 × 60 µm² (X9), focused using elliptically shaped mirrors in crossed geometry (KB-mirrors). The photon flux was 10^{10} photons/s and the acquisition time was 60s for one image at the P03. At the X9 photon flux was 10^{8} photons/s and the acquisition time was 20s for one image. A schematic representation of the experimental setups is shown in Figure 1. Bone sample acquisition was performed by extracting a 10 mm diameter cylinder of trabecular bone from the femoral head of a fresh frozen bovine femur. From the extracted cylinder, slices comprising the trabecular network were cut to 150 µm thickness and stored in the freezer at -20°C. The slices were thawed and placed in clamps immediately before examination. The samples were mounted on top of a micropositioner (X9) or a piezo-driven nanopositioner, each allowing for sufficiently precise movements of the sample with respect to the (fixed) beam. Using these setups, micro- and nanodiffraction data were recorded in a 2D scanning fashion, with step sizes (horizontal × vertical) of 1.5 × 4 µm (P03) and 40 × 100 µm (X9).

Different sample regions were chosen using the in-line video microscopes installed at the beamlines and the scanned areas ranged from 45 x 80 µm² (P03) to 100 x 400 µm² (X9), generating in this way several hundreds of diffraction images. In order to record diffraction data from one region at

different electrostatic fields (at voltages from 0V to 100V), each subsequent scan was shifted vertically by 1.5 µm (P03) and 20 µm (X9) (see Figure 3). Using this interlined scan pattern, no spot was irradiated twice, allowing for each recorded data set to remain unharmed by the radiation damage from the previous scan.

In order to enable application of the electrostatic field onto the samples in situ, i.e. while diffraction data was recorded, special sample holders were used. At P03 beamline, the sample was placed onto a 200 µm Kapton foil while in wet condition where it remained stuck to once dried. Next to the sample, two metal-foil electrodes were attached to the Kapton foil, with the sample sitting in between the electrodes but without any direct contact to them. This way, the Kapton foil acted as both, a support of the brittle sample as well as a support for the electrodes (made of aluminum foil glued to the Kapton foil). Using thin gold wires, a DC source was connected to the electrodes without inducing mechanical strain onto the sample holder and data were recorded at 0V, 5V and 10V voltage and an electrode spacing of 4 mm, yielding a maximum electrostatic field of 2,500 V/m. For the experiment at X9, on the other hand, a plastic sample holder was manufactured using 3D printing with metal electrodes incorporated into the holder and voltages of 0V, 50V and 100V were used at an electrode distance of 15 mm, i.e. an electric field of 6,666 V/m.

The data of the different samples are summarized in table 1.

The diffraction data were recorded with typical acquisition times of 20 seconds at X9 and 5 seconds at P03. At P03 a high resolution 2D CCD detector (Imagestar 9000, Photonic Science Ltd., Robertsbridge, UK) was used, having an input size of 188.2 × 188.2 mm² and a pixel size of 93.3 µm, at a sample-to-detector distance of 321,3 mm [16]. At X9, two detectors were used simultaneously, where the SAXS data was recorded using a Dectris Pilatus 1M detector (pixel size 172µm) at a distance of 7.9 m while the WAXS data was recorded using a canted Photonics Science CCD detector at 0.3 m [18]. The sample-to-detector distances as well as the beam center positions and detector tilts were determined using LaB_6 and Silver Behenate powder calibration standards.

In our experiments the detector had to be placed at a sufficient large distance from the sample in order to increase the resolution on the detector in order to observe shifts with high accuracy. As a consequence the two dimensional WAXS pattern was not completely recorded by the detector. Therefore, the small angle scattering signal had to be used to determine the orientation of the HA crystals, while the lattice constant could be determined by the WAXS evaluation.

In the small angle scattering region in principle two scattering signal can be detected one originating from the collagen matrix and the other one from the HA crystals. However, the small angle scattering signal of the inorganic part is strong compared to the organic collagen matrix and is, therefore, overlapped by it. Thus, a precise structural determination of the collagen matrix is not feasible.

2.1 Data treatment

The intensity distribution in an X-ray diffraction pattern is basically a Fourier transformed representation of the electron density variation in the irradiated volume. The SAXS signal is correlated with density variations on nm-µm length scales while the WAXS signal is sensitive down to interatomic distances (i.e. lattice spacings). In our data the SAXS pattern originates from the different phases of the hydroxyapatite (HA) crystals embedded in the collagen matrix [19]. Due to fact that HA crystals in bone have a anisotropic structure (most likely plate like) [19-27], the SAXS scattering signal has a two-dimensional anisotropic shape and can be used to extract information like orientation of the HA plates and the fraction of oriented HA crystals [27]. By evaluating the two dimensional scattering signal the T parameter (percentage of orientation) and the orientation itself can be estimated.

The XRD signal is sensitive to crystal structure of HA itself. By determining the positions θ of the Bragg reflections, where θ denotes the scattering angle, the distance d of the lattice planes can be determined via the Bragg equation [28]

$$n\lambda = 2\,\mathrm{d}\,\sin\theta,$$

where λ is the X-ray wavelength and *n* is the order of the reflection. For HA the strongest Bragg reflection connected to the c-axis of the crystal originates from the 002 lattice plane which is perpendicular to the long axis of the plate like HA crystals. This reflection was used for the data evaluation.

The determination of the peak position was done by fitting a combination of a Gaussian and linear function, which accounted for the background. In order to determine the size of the error which might come e.g. from an incorrect fitting of the peak position due to a shift of the background 20 positions at each voltage were averaged and the mean square error was determined. A typical integrated scattering pattern is shown in the supporting information in figure 1.

The actual spacing of the HA lattice planes can be influenced by stress acting on the collagen matrix and thereby being transferred onto the embedded crystals. A compressive stress in the crystal can reduce the lattice spacing as the lattice planes are pushed closer together. The strain can be calculated via $\varepsilon = \frac{d_0 - d}{d_0}$, where d_0 denotes the strain free lattice constant and d the lattice constant with strain [29]

3. Results

31. Sample 1.

The effect of electric field on trabecular bone was first studied with respect to the orientation of the hydrxylapatatite and the collagen matrix. Voltages of 0V, 50V and 100V were applied resulting in electric fields of 0 V/m, 3,333 V/m and 6,666 V/m, respectively. Two different positions (1P1, 1P2) in the same bone sample were investigated, with position 1P2 containing hydroxyapatite crystals mainly oriented along the field gradient. At the other position 1P1, the HA crystals were orientation mainly perpendicular to the electric field gradient sample. At either position data was recorded from a 2D array of 20 spots, with one such set for each voltage. Care had to be taken to avoid beam damage due to the x-ray beam and so the scan patterns were shifted slightly with respect to each

other, as mentioned in the previous section and shown in figure 2. The orientation of the HA crystals, the relative fraction of oriented material and the positon of the Bragg reflection from the 002 hydroxyapatite lattice plane were determined and averaged for each voltage. At the position 1P1 60% of the HA is oriented with an angle of 80° to the electrical field, whereas at position 1P2 60% of the HA crystals are oriented by 20° with respect to the electrical field. The plots of the orientation and the relative fraction of orientated material at each probed position show that no significant variation for these parameters is observable at different electric fields, see figure supporting information S1 and S2.

Figure 3 shows the average value of the 002 hydroxyapatite Bragg reflection at different applied voltages and orientations. The error bars were determined by the standard deviation of the averaged Bragg reflection position of the 20 single positions. The data indicates that for an orientation of HA parallel to the electric field the lattice constant remains constant up to the highest electric field strength of 6,666 V/m. This is different at the position were the HA is oriented perpendicular to the electric field. Here a total shift of $\Delta q = 0.021\ nm^{-1}$ occurs at an electric field of 6,666 V/m. This shift of the Bragg reflection corresponds to an induced strain of $\varepsilon = 9 * 10^{-4}$ in the HA crystals by the collagen matrix.

3.2 Sample 2.

Furthermore, to shed light on the effect of the electric field on the microstructure of trabecular bone, a second experiment at the Nanofocus Endstation of the P03 with a much smaller beamsize was performed (1.5µm x 1.5µm). A mesh with the size of 80 µm x 45 µm was probed, giving information on micro meter resolution with 600 locations at each position.

Figure 4 shows a microscope image of the bone sample after the measurement. The two different positions (1 & 2) which were scanned are clearly visible. A sketch shows the orientation of the electric field. This image also shows the beam damage which was induced by the highly focused X-ray

beam. As mentioned earlier the scans of each voltage were shifted by 1.5µm, thus, no spot was irradiated twice.

In the following the observations made at position 1 (sample 2P1) will be demonstrated. The position of 002 scattering reflection of the hydroxyapatite crystals at zero voltage was determined from the diffraction patterns at each position of the scanned mesh and the lattice constant was calculated. Two dimensional maps showing the spatial variation of the lattice constant over the probed area are shown in figure 5a along with a histogram (figure 5d) showing the number of occurrence of each lattice constant. The two dimensional map shows a clear distribution of lattice constants in the bone. The lower region which belongs to the edge of the bone exhibits a smaller lattice constant than the inner part of the bone. The histogram indicates an accumulation of the lattice constants at 3.330 Å and at 3.326 Å. The total variation in the lattice constant over the whole probed area is 0.01 Å. Figure 6 shows the orientation of the HA crystals indicated by the arrows and the percentage of oriented crystals by the colour code. The yellow and red areas indicate a high degree of orientation and are distributed at the bottom and top part of the probed area. However, the middle part shows only a minor degree of orientation in the cutting plane. The main orientation in this part of the bone is in the x-direction.

By comparing the map showing the orientation of the HA crystals with the map of the distribution of the lattice constant some similarities can be observed. The occurrence of a lower lattice constant seems to be connected to a higher degree of orientation.

The two dimensional map for the electric field of 1,500 V/m and 2,500 V/m are shown in figure 5b and 5c. The map reveals that the lattice constant decreases as the voltage is increased to 2,500 V/m. This is in line with the observations in the foregoing experiment. The histogram shows also the distribution for the different electric field strengths. A gradual increase with the voltage can be observed. The main lattice constant shifts from 3.332 Å to 3.331 Å for 1,250 V/m to finally 3.330 for 2,500 V/m. Thus the lattice constant shifts by 0.002 Å as the electric field strength is increased by

1250 V/m. From this shift an average induced strain in the bone matrix of $\varepsilon = 6 * 10^{-4}$ can be calculated.

The evaluated parameters of the position 2P2 are summarized in Figure 7. The lattice constant distribution at 0 V/m is depicted in figure 7a. The spatial distribution of the lattice constant is not as pronounced as at position 2P1. This hints at a more homogeneous structure in this part of the bone. The histogram for the lattice constant which is depicted in figure 7d is centred at 3.330 Å. By looking at the degree of the orientation it can be seen that the hydroxyapatite crystals are much more aligned along the direction of the trabecula, see figure 8 and figure 6. Taken together, at position 2 the HA crystals are much more homogenous aligned. The orientation is mainly 45° with respect to the electric field gradient.

The histogram of the lattice constants reveal that there is no change of the lattice constant (or that it is non-detectable) at field strengths of 1,250 V/m and 2,500 V/m (figure 7d). This can also be seen in the two dimensional maps which is depicted in figure 7n and 7c. The small deviation for each voltage could be due to the shift of 1.5 µm for each voltage.

4. Discussion & Conclusion

Bone is a hierarchical structured material which is mainly composed of hydroxyapatite crystals and a collagen matrix. The stability and elasticity of the bone is a result of the interplay of these two constituents, with a specific dependence on their orientations and alignments. It is noteworthy that the structure of the HA crystals can vary depending on different factors. Residual stress is not the only cause for lattice spacing modulations: non-stoichiometric compositions and the exchange of hydroxyapatite and carbonate apatite can also vary the crystal structure [30, 31]. This explains why the crystal structure varies between samples and even in between positions of the same sample. Relative variations of the lattice spacing, however, can be detected with an accuracy of 0.1 % using X-ray diffraction methods.

In this study the inverse piezo electric effect in trabecular bones was investigated. To achieve this, lattice spacing changes of the HA crystals were studied in their native state i.e. embedded in the collagen matrix and at different applied electric field strengths. Different positions in the trabecular network were probed.

Because hydroxyapatite is a centro symmetric crystal the material cannot exhibit piezo electric properties [32, 33]. Therefore, the observed inverse piezo electric effect is likely to be mediated by the other main constituent of trabecular bone: the collagen matrix. This assumption is supported by investigations on macroscopic samples where a piezo electric voltage was detected if shearing forces acted on the collagen fibrils [14, 34]. Also, investigations on isolated collagen fibrils showed that an electrical field perpendicular to the axial orientation of the collagen can introduce the shear deformation [32].

Structural investigations of collagen by x-ray crystallography show that the super lattice of collagen is formed by a quasihexagonally packing of single collagen molecules.[35] Molecular dynamic simulations indicate that the piezo electric response originates from compression of the helix by the electrical field. [36]This explains why collagen only exhibits a shear piezo response. In the symmetry group, C_6, the inverse piezo electric tensor is, [14, 37]

$$\begin{pmatrix} \varepsilon_{11} \\ \varepsilon_{22} \\ \varepsilon_{33} \\ 2\varepsilon_{23} \\ 2\varepsilon_{13} \\ 2\varepsilon_{12} \end{pmatrix} = \begin{pmatrix} 0 & 0 & d_{13} \\ 0 & 0 & d_{31} \\ 0 & 0 & d_{33} \\ d_{14} & d_{15} & 0 \\ d_{15} & -d_{14} & 0 \\ 0 & 0 & 0 \end{pmatrix} \begin{pmatrix} E_x \\ E_y \\ E_z \end{pmatrix}$$ The constant d_{ii} are the piezoelectric constants, ε_{ii} are the strain and E_i are the electric field components. The z-axis of the coordinate system is oriented parallel to the axis of the collagen fibril. The x and y axis are perpendicular to the fibril. If the collagen fibril is placed in an homogenous electric field, with only a component along E_x (E_y = 0 and E_z=0) being perpendicular to the fibril axis. Therefore, the only contribution being $2\varepsilon_{23} = d_{14}E_x$ and $2\varepsilon_{13} = d_{15}E_x$ inducing a shear response perpendicular to the fibril axis, thus, a deformation along the axis.

This highlights the importance of the orientation of the collagen network to the electrical field. In our experiments we did not make an *a priori* selection of specific orientations of the collagen network.

"The preferred oration of the helical axis of collagen and HA crystals was observed to parallel. But the distribution of the orientation is broader for collagen than for HA crystals, which are better align parallel.[27, 38, 39] Nevertheless, the orientation of the collagen can be deduced from the orientation of the hydroxyapatite crystals as the long axis of the HA crystals coincides with the mean orientation of the collagen [27, 38]. Thus by determining the orientation of HA crystals the orientation of the collagen can be derived indirectly.

Our first set of experiments clearly indicates that the collagen network is able to induce stress onto the the HA crystals, therby causing a detectable strain. For the first sample 1P1, where HA crystals and with it the collagen network is oriented perpendicular to the electric field, a shift of the HA 002 lattice constant can be observed which results from a significant strain. In the second position 1P2 were the collagen is oriented mostly parallel no shift of the lattice constant can be seen. The stress which was observed achieved a value of $\varepsilon = 9 * 10^{-4}$.

In the second experiment another sample was investigated with higher spatial resolution at two positions (2P1 and 2P2). The samples investigated showed a lateral distribution of the lattice constant across the sample itself. This shows the inhomogeneous nature of the hydroxyapatite on a micrometre scale across the bone itself. At position 2P1, i.e. where the main orientation was perpendicular to the electric field, two clearly separated regions were detected, each having a rather homogeneous lattice spacing. The occurrence of a higher degree of orientation coincides with a higher lattice constant. A strain of $\varepsilon = 6 * 10^{-4}$ was calculated which is in the same magnitude as for the first sample. For position 2P2 no deformation is observed as the collagen matrix is oriented with an angle of 40° to 60° with respect to the electric field. A part of the electric field will couple to the collagen but due to the angle the electric field component is heavily reduced or the deformation is

undetectable within the error bounds of our data. Either way, no shift of the lattice constant was observed.

In conclusion, it could be shown that a compression of the HA unit cell can in fact be accomplished by the application of an adequately aligned electrical field to the bone, i.e. if the HA crystals and with it the collagen matrix are aligned perpendicular to the electrical field. Our data indicates that if collagen is oriented perpendicular to the electric field, a polarisation in the collagen matrix can be induced and the collagen is sheared as a consequence. The shear strain is transferred onto the HA crystals which are deformed as a consequence, indicated by the observed smaller lattice constant. On the other hand, if collagen is oriented parallel no polarization is observed and no detectable strain is exerted on the HA crystals.

In the application of bone growth stimulation typical field strengths of 5 to 70 V/m are recommended[4]. Extrapolating the effects detected in our experiment down to this field strength a strain of $\varepsilon = 1 * 10^{-5}$ can be estimated for these applications. Studies report that osteocytes are able to sense strain in bone down to $\varepsilon = 8 * 10^{-6}$ [40-42]. Bone is a complex structure where different process and mechanisms are involved and electric fields can have many different influences. Our data provides strong evidence that the inverse piezo electric effect in bone could induce enough strain which the bone cells can sense and thereby is a mechanism which can trigger bone growth.

Acknowledgements

The nanofocus endstation was equipped through financial support by the German Federal Ministry of Education and Research (BMBF projects 05KS7FK3 and 05K10FK3) which is greatly acknowledged. We thank the P03 team for provision of the beam to the nanofocus end station. Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-98CH10886. Dr. Lin Yang is acknowledged for his help during beam time at NSLS beamline X9. We acknowledge the virtual institute VH-VI-523 and DFG graduate school Welisa 1505/1-2 for financial support.

Tables

Table 1: Summary of the different samples tested. Within each of the two synchrotron experiments data were recorded from one bone sample but different position were investigated, i.e. the bone was oriented along different orientations with respect to the electric field.

	Orientation of the HA crystals to the electrical field	Experimental run	resolution	Beamline	Bone sample
Sample 1P1	Perpendicular	1	40µm	X9, NSLS	Sample 1
Sample 1P2	Parallel	1	40µm	X9 NSLS	Sample 1
Sample 2P1	Perpendicular	2	1.5µm	P03, PETRA III	Sample 2
Sample 2P2	parallel	2	1.5µm	P03, PETRA III	Sample 2

Captions

Figure 1 Schematic representation of the scanning X-ray microdiffraction experiment performed at P03 beamline. The monochromatic beam, as delivered by the beamline, was focused using a pair of elliptical mirrors. The sample was positioned at the focal plane and inside a homogenous electrostatic field. While the sample was scanned across the beam in small steps, diffraction images were recorded at each individual position. A subsequent automated processing of the recorded data evaluated it's the SAXS and WAXS portions and delivered a spatially resolved map of hydroxyapatite orientation and lattice spacing.

Figure 2 Close-up of the sample environment in the experimental setup at beamline X9 (left). The sketch shows the direction of the electric field. (right) Schematic representation of the interlined scan pattern used to record data at each position of sample 1

Figure 3 Averaged Bragg position of the 002 hydroxyapatite lattice reflection at different electric field gradients and orientation in sample 1 determined by the evaluation of the SAXS pattern.

Figure 4 (left) Light microscope image of the bone sample 2 after the experiment at P03 beamline, with the scanned regions clearly visible as dark rectangles (45 x 80 µm)

Figure 5 lattice constant distribution in sample 2 determined by the evaluation of the WAXS pattern

, at position 2P1. Two dimensional distribution at an electric field of a) 0 V/m. b) 1,250 V/m c) 2,500 V/m) Histogram of the lattice constant for the three electric field strengths (0 V/m , 1,250 V/m, 2,500 V/m). The colour bar from the two dimensional maps is placed and scaled to the x-axis of the histogram.

Figure 6 Orientation and degree of orientation at position 2P1 determined by the evaluation of the SAXS pattern. The color code indicates the percentage of oriented HA crystals whereas the arrows indicate the orientation.

Figure 7 Lattice constant distribution in sample 2 determined by the evaluation of the WAXS pattern, at position 2P2. Two dimensional distribution at an electric field of a) 0 V/m. b) 1,250 V/m c) 2,500 V/m) Histogram of the lattice constant for the three electric field strengths (0 V/m , 1,250 V/m, 2,500 V/m). The colour bar from the two dimensional maps is placed and scaled to the x-axis of the histogram.

Figure 8 Orientation and its degree at position 2P2 determined by the evaluation of the SAXS pattern. The colour code indicates the percentage of oriented HA crystals whereas the arrows indicate the orientation.

[1] Goodwin CB, Brighton CT, Guyer RD, Johnson JR, Light KI, Yuan HA. A double-blind study of capacitively coupled electrical stimulation as an adjunct to lumbar spinal fusions. Spine 1999;24:1349-56.
[2] Brighton CT, Black J, Friedenberg ZB, Esterhai JL, Day LJ, Connolly JF. A Multi-Center Study of the Treatment of Non-Union with Constant Direct-Current. J Bone Joint Surg Am 1981;63:2-13.

[3] Diniz P, Shomura K, Soejima K, Ito G. Effects of pulsed electromagnetic field (PEMF) stimulation on bone tissue like formation are dependent on the maturation stages of the osteoblasts. Bioelectromagnetics 2002;23:398-405.
[4] Kraus W. Magnetic-Field Therapy and Magnetically Induced Electrostimulation in Orthopedics. Orthopade 1984;13:78-92.
[5] Mittelmeier W, Lehner S, Kraus W, Matter HP, Gerdesmeyer L, Steinhauser E. BISS: concept and biomechanical investigations of a new screw system for electromagnetically induced internal osteostimulation. Arch Orthop Traum Su 2004;124:86-91.
[6] Brighton CT, Wang W, Seldes R, Zhang GH, Pollack SR. Signal transduction in electrically stimulated bone cells. J Bone Joint Surg Am 2001;83A:1514-23.
[7] Fassina L, Visai L, Benazzo F, Benedetti L, Calligaro A, De Angelis MGC, et al. Effects of electromagnetic stimulation on calcified matrix production by SAOS-2 cells over a polyurethane porous scaffold. Tissue Eng 2006;12:1985-99.
[8] Fassina L, Saino E, Sbarra MS, Visai L, De Angelis MGC, Magenes G, et al. In vitro electromagnetically stimulated SAOS-2 osteoblasts inside porous hydroxyapatite. Journal of Biomedical Materials Research Part A 2010;93A:1272-9.
[9] Zhou J, Ming LG, Ge BF, Wang JQ, Zhu RQ, Wei Z, et al. Effects of 50 Hz sinusoidal electromagnetic fields of different intensities on proliferation, differentiation and mineralization potentials of rat osteoblasts. Bone 2011;49:753-61.
[10] Grunert PC, Jonitz-Heincke A, Su Y, Souffrant R, Hansmann D, Ewald H, et al. Establishment of a Novel In Vitro Test Setup for Electric and Magnetic Stimulation of Human Osteoblasts. Cell Biochem Biophys 2014;70:805-17.
[11] Ellenrieder M, Tischer T, Kreuz PC, Frohlich S, Fritsche A, Mittelmeier W. Arthroscopically assisted therapy of avascular necrosis of the femoral head. Oper Orthop Traumato 2013;25:85-94.
[12] Su YK, Souffrant R, Kluess D, Ellenrieder M, Mittelmeier W, van Rienen U, et al. Evaluation of Electric Field Distribution in Electromagnetic Stimulation of Human Femoral Head. Bioelectromagnetics 2014;35:547-58.
[13] Griffin M, Bayat A. Electrical Stimulation in Bone Healing: Critical Analysis by Evaluating Levels of Evidence. Eplasty 2011;11:34.
[14] Fukada E, Yasuda I. On the Piezoelectric Effect of Bone. J Phys Soc Jpn 1957;12:1158-62.
[15] Shamos MH, Lavine LS. Piezoelectricity as a Fundamental Property of Biological Tissues. Nature 1967;213:267-&.
[16] Krywka C, Keckes J, Storm S, Buffet A, Roth S, Döhrmann R. Nanodiffraction at MINAXS (P03) beamline of PETRA III. Journal of Physics: Conference Series 2013;425:072021.
[17] Buffet A, Rothkirch A, Dohrmann R, Korstgens V, Kashem MMA, Perlich J, et al. P03, the microfocus and nanofocus X-ray scattering (MiNaXS) beamline of the PETRA III storage ring: the microfocus endstation. J Synchrotron Radiat 2012;19:647-53.
[18] Yang L. Using an in-vacuum CCD detector for simultaneous small- and wide-angle scattering at beamline X9. J Synchrotron Radiat 2013;20:211-8.
[19] Fratzl P, Groschner M, Vogl G, Plenk H, Eschberger J, Fratzlzelman N, et al. Mineral Crystals in Calcified Tissues - a Comparative-Study by Saxs. J Bone Miner Res 1992;7:329-34.
[20] Landis WJ. Mineral characterization in calcifying tissues: Atomic, molecular and macromolecular perspectives. Connect Tissue Res 1996;35:1-8.
[21] Landis WJ, Hodgens KJ, Arena J, Song MJ, McEwen BF. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. Microsc Res Techniq 1996;33:192-202.
[22] Weiner S, Traub W. Bone-Structure - from Angstroms to Microns. Faseb J 1992;6:879-85.
[23] Fratzl P, Fratzlzelman N, Klaushofer K. Collagen Packing and Mineralization - an X-Ray-Scattering Investigation of Turkey Leg Tendon. Biophys J 1993;64:260-6.
[24] Fratzl P, Fratzlzelman N, Klaushofer K, Vogl G, Koller K. Nucleation and Growth of Mineral Crystals in Bone Studied by Small-Angle X-Ray-Scattering. Calcified Tissue Int 1991;48:407-13.

[25] Fratzl P. Small-angle scattering in materials science - a short review of applications in alloys, ceramics and composite materials. J Appl Crystallogr 2003;36:397-404.
[26] Paris O, Zizak I, Lichtenegger H, Roschger P, Klaushofer K, Fratzl P. Analysis of the hierarchical structure of biological tissues by scanning X-ray scattering using a micro-beam. Cell Mol Biol 2000;46:993-1004.
[27] Rinnerthaler S, Roschger P, Jakob HF, Nader A, Klaushofer K, Fratzl P. Scanning small angle X-ray scattering analysis of human bone sections. Calcified Tissue Int 1999;64:422-9.
[28] Bragg WH, Bragg WL. The reflexion of x-rays through crystals. Z Anorg Chem 1914;90:169-81.
[29] Hauk V. Structural and Residual Stress Analysis by Nondestructive Methods. Elsevier 1997.
[30] Almer JD, Stock SR. Internal strains and stresses measured in cortical bone via high-energy X-ray diffraction. J Struct Biol 2005;152:14-27.
[31] Handschin RG, Stern WB. Crystallographic Lattice Refinement of Human Bone. Calcified Tissue Int 1992;51:111-20.
[32] Minary-Jolandan M, Yu MF. Nanoscale characterization of isolated individual type I collagen fibrils: polarization and piezoelectricity. Nanotechnology 2009;20.
[33] Minary-Jolandan M, Yu MF. Nanomechanical Heterogeneity in the Gap and Overlap Regions of Type I Collagen Fibrils with Implications for Bone Heterogeneity. Biomacromolecules 2009;10:2565-70.
[34] Hastings GW, Mahmud FA. Electrical Effects in Bone. J Biomed Eng 1988;10:515-21.
[35] Orgel JPRO, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. P Natl Acad Sci USA 2006;103:9001-5.
[36] Ravi HK, Simona F, Hulliger J, Cascella M. Molecular Origin of Piezo- and Pyroelectric Properties in Collagen Investigated by Molecular Dynamics Simulations. J Phys Chem B 2012;116:1901-7.
[37] Kolosov O, Gruverman A, Hatano J, Takahashi K, Tokumoto H. Nanoscale Visualization and Control of Ferroelectric Domains by Atomic-Force Microscopy. Phys Rev Lett 1995;74:4309-12.
[38] Nakano T, Kaibara K, Tabata Y, Nagata N, Enomoto S, Marukawa E, et al. Unique alignment and texture of biological apatite crystallites in typical calcified tissues analyzed by microbeam X-ray diffractometer system. Bone 2002;31:479-87.
[39] Sasaki N, Sudoh Y. X-ray pole figure analysis of apatite crystals and collagen molecules in bone. Calcified Tissue Int 1997;60:361-7.
[40] Kunnel JG, Gilbert JL, Stern PH. In vitro mechanical and cellular responses of neonatal mouse bones to loading using a novel micromechanical-testing device. Calcified Tissue Int 2002;71:499-507.
[41] Kunnel JG, Igarashi K, Gilbert JL, Stern PH. Bone anabolic responses to mechanical load in vitro involve COX-2 and constitutive NOS. Connect Tissue Res 2004;45:40-9.
[42] Fermor B, Gundle R, Evans M, Emerton M, Pocock A, Murray D. Primary human osteoblast proliferation and prostaglandin E-2 release in response to mechanical strain in vitro. Bone 1998;22:637-43.