Supporting Information for

Multiscale characterization of pathological bone tissue

E. Deniz Eren1, Wouter H. Nijhuis2, Freek van der Weel1, Aysegul Dede Eren3,4, Sana Ansari3,5, Paul H.H. Bomans1, Heiner Friedrich1,3, Ralph J. Sakkers2, Harrie Weinans2,6 and Gijsbertus de With1*

1Laboratory of Physical Chemistry, Department of Chemical Engineering and Chemistry, Eindhoven University of Technology, Eindhoven, The Netherlands

2Department of Orthopaedic Surgery, University Medical Centre Utrecht, Wilhelmina Children’s Hospital, The Netherlands

3Institute for Complex Molecular Systems (ICMS), Eindhoven University of Technology, P.O. Box 513, 5600 MB Eindhoven, The Netherlands

4Eindhoven University of Technology, Department of Biomedical Engineering, Biointerface Science, Eindhoven, the Netherlands.

5Orthopaedic Biomechanics, Department of Biomedical Engineering, Eindhoven University of Technology, PO Box 513, 5600 MB, Eindhoven, the Netherlands

6TU Delft, Department of Biomechanical Engineering, Delft, the Netherlands

*Correspondence to: G. de With (g.dewith@tue.nl; orcid.org/0000-0002-7163-8429)

This PDF file includes:

Appendix 1 to 14
Captions for Supplementary Movies S1 to S8
References for Supplementary Information document

Other supplementary material for this manuscript include the following:

Supplementary Movies S1 to S8
Appendix 1: Sample information

| Bone Type | Age of the individuals | Gender of the Individuals | Reason |
|-----------|------------------------|---------------------------|--------|
| OI Type IV | 8                      | Female                    | COL1A2 gene (in position 754 there is a glycine substitution with serine) |
| SEDC      | 6                      | Female                    | COL2A1 a gene that provides instructions for the production of the pro-alpha1(II) chain of type II collagen |

Supplementary Table S1. Bone samples used in this study.

Appendix 2: Materials and Methods

Sample Preservation

1. Cleaning

Samples collected from the patient first were cleaned before use in the subsequent preparation and characterization steps. This cleaning step makes certain that no blood, unwanted cells or other biological material is present during subsequent processing and imaging steps.

Aqueous phosphate-buffered saline (PBS) solution is used for the washing step to prevent the samples from decomposing, it prevents demineralization from taking place before fixation, while the neutral pH of the PBS ensures that the collagen remains stable and the aqueous nature of the solution results in no dehydration of the bone. Therefore the PBS’s neutral pH and ion concentration create an environment, which ensures that no degradation takes place\(^1\).

2. Fixation

After the initial cleaning, the samples were further processed. These subsequent steps ensure that the samples can be prepared, characterized, and stored for long time periods. The first step in this process is fixation, its purpose is the preservation of the chemical and physical structure in subsequent processing within sample preparation steps and usage during characterization. Fixation is a physicochemical process that is gradual and complex, involving...
diffusion of fixative into the tissue and a variety of potential physical and chemical reactions. Currently, there is no ideal fixative and therefore there are 4 groups of agents that are primarily used: Aldehydes, oxidizing agents, alcohol-based fixatives, and metallic fixating agents. The choice of agent depends on the type of tissue and experiments that are to be performed\(^\text{2}\).

During the sample preparation, fixation was performed before and after the demineralization. Both fixatives used in this study are an aldehyde type (paraformaldehyde and glutaraldehyde) and the formation of crosslinks occurs via a reaction with the amino acids present on cells and most importantly the collagen. The goal of the fixation, for the bone samples, is to preserve the hierarchical structure of the collagen when the sample is demineralized, processed, and stored under different conditions. The first step uses paraformaldehyde (PFA), being a small molecule it has an ability to quickly diffuse into the sample. The initial binding reaction is largely completed within 24 hours. However, the disadvantage is that the following crosslink reaction is quite slow and form only over short distances between collagen chains, due to the PFA being a short/small molecule. PFA mostly binds to the primary amine of the lysine and the secondary amine on the peptide linker of collagen, but there are other crosslink possibilities\(^{3,4}\).

\[\begin{align*}
R_1 & \quad R_2
\end{align*}\]

\[\begin{align*}
\text{R}_1 \quad \text{NH} & \quad \text{+ CH}_2(\text{OH})_2 \\
\text{R}_2 & \quad \text{R}_1 = \text{R} \\
\text{R}_2 & \quad \text{R} / \text{R} \\
\text{R}_1 & \quad \text{R}_2 \quad \text{N-CH}_2\text{OH} \\
\text{R}_1 & \quad \text{R}_2 \quad \text{N-CH}_2\text{OH}
\end{align*}\]

**Supplementary Figure S1.** The crosslinking reaction of paraformaldehyde with the most common structures present on the amino acids of the Type 1 Collagen chain.

To ensure the structure remains rigid after demineralization a second fixation step is performed. Glutaraldehyde (GA) is used in this second step; it diffuses into the tissue at a slower rate than PFA due to its relatively larger size. However, the benefit is that
glutaraldehyde has a higher crosslink potential. It contains two reactive groups and can form the crosslink over a longer distance due to its relatively larger size. Furthermore, glutaraldehyde is able to form small oligomers/polymers with reactive aldehyde groups that can react with the collagen and bridge an even longer distance. Crosslinks are formed between the amino groups present on amino acids, the aldehyde group of glutaraldehyde reacts with $\varepsilon$-amine groups of lysine or hydroxylysine present in collagen.

The combination of paraformaldehyde and glutaraldehyde uses the quick penetration of the PFA to start the stabilization of the structure. The slower penetrating glutaraldehyde used in the second step then forms additional crosslinks bridging larger gaps making it more rigid and solid. This fixation procedure preserves the native structure of bone as well as possible.

![Supplementary Figure S2.](image)

a) The polymerization reaction of glutaraldehyde, an aldehyde side chain is formed on each unit. b) The crosslinking reaction of the glutaraldehyde polymer with the amino group of collagen.

3. Demineralization

Demineralization of the native bone tissue is an important step in the sample preparation and the characterization techniques, such as polarized optical microscope (POM) and Scanning
electron microscope/focused ion beam serial slice and view (SEM/FIB SSV). Removal of the minerals reduces interferences and, therefore, provides improved contrast. However, there are also other benefits for further sample preparation steps, e.g. in the use of microtomy. The removal of the minerals leads to a reduced hardness of bone, which allows the samples to be cut more easily and, therefore, results in fewer or no artifacts during microscopy.

Demineralization of the native bone samples is performed using a demineralizing agent, which is dissolved in a suitable solution. There are two types of chemical groups that are primarily used in the demineralization of mineralized tissue, acids, and chelating agents. Despite that they are from two different chemical groups, the reaction that takes place within bone tissue is quite similar. The carbonated hydroxyapatite (cHAP) in the mineralized tissue has a natural reversible dissociation reaction when submerged in an aqueous medium. If nothing were to be added to this aqueous solution an equilibrium would be reached. However, the addition of the acid or chelating agent disturbs this equilibrium, which results in further demineralization of the tissue until a new equilibrium is reached.

Previous studies have shown that using acid as the demineralizing agent leads to poorer preservation of the tissue relative to the chelating agent, which is partly due to the low pH at which the demineralization is carried out when using an acid. The low pH has a degrading and corrosive effect on the collagen that lead to changes in the structure\(^5\). The degradation of the collagen is an undesired effect, due to the interest in the changes that occur in the collagen structure in pathological tissues. Therefore, a chelating agent was used during the demineralizing, specifically ethyl diamine tetraacetic acid (EDTA).
Supplementary Figure S3 shows how the chelating agent, EDTA, disrupts the equilibrium of cHAP in an aqueous medium due to the formation of a complex with the calcium that is being released. The complex formation with calcium results in the equilibrium of the dissociation reaction shifting to the right. This continues as long as there is a non-complexed EDTA present in the solution. From the reaction mechanism, it can also be concluded that the pH would still be lowered due to the release of protons from the EDTA when binding to the calcium \(^6,7\). However, monitoring the pH, regularly replacing the solutions, and starting around a neutral pH, which is near the optimal pH for the reaction, should result in the inability of pH to influence the collagen structure.

An atomic absorption spectrometer (AA-7000 / Shimadzu) was used to characterize the calcium content of the bone samples. All samples were measured using the continuous flame mode. Samples were diluted with ultra-pure water depending on what was necessary to bring the concentration within the concentration range that is known to give a linear relation for the absorbance versus concentration curve (0-4 ppm).

By employing Atomic Absorption Spectrometry (AAS) one can determine the concentration of calcium in the solution for a specific volume, which can be correlated to the amount of released mineral from each sample. Initial experiments were conducted at 5%,
10%, and 20% ethylenediaminetetraacetic acid (EDTA) concentrations to determine the optimum time for a complete demineralization (Figure S4). The results indicate that the optimum time for a complete demineralization is about 2 weeks when using 5% EDTA (Figure S4a). As expected, the necessary time for a complete demineralization can be reduced by increasing the concentration of EDTA (Figure S4b), however, owing to concerns regarding the preservation of the native bone structure, only 5% EDTA was used unless indicated otherwise.

**Supplementary Figure S4.** a) A control measurement for the 20% concentration. The time till complete demineralization increases with a heavier sample. Samples were diluted by a factor 100. b) The cumulative calcium concentration in ppm for different demineralization agent concentrations. Samples were diluted by a factor of 100.
Supplementary Figure S5. Cumulative calcium concentration for OI type IV and SEDC bone in the solution per day (normalized to the same weight of the OI type IV and control bone pieces).

4. Staining

Constant improvement of the SEM characterization method has led over time to an increasing interest from the biological community to characterize their samples with this method. Unfortunately, most biological materials are not suited for this method due to their high resistivity (bone: $10^8 - 5 \times 10^{10}$ Ω cm$^{-1}$), which leads to significant charging when it is exposed to the electron beam. These problems could be solved when using low accelerating voltages, the BSE detector, or short exposure times, but these restrictions are undesirable and lead to poor image contrast$^8$. This in turn may obscure small or low contrast features and, therefore, leads to less accurate results relative to when the full range of imaging settings can be employed$^9$.

Bone also requires staining to make it suitable for characterization with SEM and
performing SSV measurements. Multiple methods are available that can be used in staining for SEM, such as deposition of a small metal layer with a sputter coater or using a negative staining agent that covers the material with heavy metal. However, both methods lack the needed penetration depth of the agent into the sample and can cause significant sample shrinkage. Therefore, these methods are deemed insufficient and/or suboptimal for SSV measurements\(^{(10)}\).

To stain the complete sample including the inner laying structures OTOTO heavy metal staining was chosen. OTOTO can penetrate into the bone, fixate the structure, and improve the contrast. The O and T represent the chemicals osmium tetroxide (OsO\(_4\)), staining agent, and thiocarbohydrazide (TCH), linking agent. The OTOTO abbreviation already suggests that the sample is subjected to alternating solutions of osmium and TCH, creating chains on the sample. During this process it is very likely that in addition to the formation of single chains there will also be chains that are connected between two sample surfaces and therefore acting as a fixative, contributing to further fixation of the bone samples\(^{(11)}\).

The contrast generated in the sample is due to the osmium tetroxide reacting with the material and deposition onto and into the material. Previous studies have shown that osmium tetroxide binds itself to the sample via the formation of a cyclic ester with unsaturated hydrocarbons on lipids and nucleic acids\(^{(12)}\). The initial addition of OsO\(_4\) should be sufficient as for SSV, however, the additional TOTO procedure has proven to be advantageous in improving the contrast such that for example microvilli on a larva can be imaged\(^{(9)}\).

5. **Picrosirius Red Staining**

Distinguishing the orientation of the collagen fibrils in the twisted plywood, and the ordered from the disordered material is difficult even with SEM and therefore not obtainable in POM
without a staining agent. Picrosirius red stains collagen due to a strong binding interaction of the acidic sulfonic groups, on the staining agent, with the basic groups of the amino acids present in collagen. The staining agent also permits birefringence due to the parallel alignment of the dye molecules with the long axis of each collagen molecule, this would allow the orientation of the collagen fibers to be determined\(^{(13,14)}\). The colors of the stained sections range from green to red under the polarizing microscope, which according to literature depend on, the orientation, the thickness of the section, and the packing of the collagen fibers\(^{(13–15)}\). If the data shows the mentioned differences, then the images will possibly show any differences in the long-range orientation of the collagen fibrils. Picrosirius Red Staining (Connective Tissue Stain, Abcam) was used to stain collagen. In brief, bone samples prepared with sucrose incubation were embedded in Tissue-Tek (Sakura Finetek USA Inc, 25608-930), fixed to the cutting base plate of a cryotome (Leica CM1950) and longitudinally sectioned into slices with a thickness of about 5 µm. Thereafter, the optimal cutting temperature compound (Agar Scientific, AGR1180) was washed in ultra-pure water for two minutes and incubated in a Picrosirius Red solution for 60 min. Thereafter, tissue sections were first rinsed in 0.5 % acetic acid and then rinsed in absolute ethanol. Finally, the sections were dehydrated in increasing series of ethanol (25 %, 50 %, 75 % and 100 % each for 3 h and mounted in DPX mounting medium (Sigma Aldrich).

6. Plastic Embedding

The OTOTO staining leaves the sample very brittle especially after drying, therefore a plastic embedding is necessary. The embedding helps in meeting the required hardness that is necessary for the bone piece to be sectioned with a microtome. Without plastic embedding, the structure of the bone would be damaged when thin sectioning. The damage would then
possibly be incorrectly interpreted as changes in the structure of the bone and lead to incorrect results\(^{(16)}\).

Different types of epoxy and acrylate resins available can be used for plastic embedding. In this study an “Epon embedding” was used. Epon, however, is a product name for Shell its epoxy resins, and therefore it is an epoxy resin embedding. This type of embedding for electron microscopy was introduced by Kushida et al.\(^{(17)}\) in 1959, followed by Finck et al.\(^{(18)}\) (1960) and Luft et al.\(^{(19)}\) (1961), in their protocol Epon812 (Shell) was used. The interest in epoxy resins over methyl acrylate as an embedding medium is the reduced damage due to polymerization while keeping the excellent fine structure of the samples. Furthermore, the epoxy resin is also more resistant to the irradiation of the electron beam during measurement\(^{(19)}\). After epon embedding and microtoming the sample is ready to be characterized with the chosen imaging methods.

7. Statistical Tests

For a \(t\)-test we need:

\[
t_{\text{exp}} = \frac{x_1 - x_2}{s_\Delta}, \quad S_\Delta = \sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}} \quad \text{and} \quad \nu = \frac{(s_1^2/n_1)^2 + (s_2^2/n_2)^2}{\left[(s_1^2/n_1)^2/(n_1-1) + (s_2^2/n_2)^2/(n_2-1)\right]}
\]

with \(t_{\text{exp}}\) the normalized difference between the averages \(\bar{x}_1\) and \(\bar{x}_2\) with sample standard deviations \(s_1\) and \(s_2\) for \(n_1\) and \(n_2\) data points, \(S_\Delta\) the combined standard deviation and \(\nu\) the number of degrees of freedom. The null hypothesis \(H_0\) is \(\bar{x} - \bar{y} = 0\), which is true if \(t_{\text{exp}} < t_{\nu,\alpha}\). Here \(t_{\nu,\alpha}\) is the theoretical \(t\)-value for \(\nu\) degrees of freedom at significance level \(\alpha\). For the latter we use \(\alpha = 0.05\), in combination with a two-sided confidence interval. The data shows for both the twisted plywood periodicity (Table S2) and the canaliculi diameter (Table S3) that \(t_{\text{exp}} > t_{\nu,0.05}\), therefore the null hypothesis is rejected and a significant difference between control bone and the OI bone samples exists.
Supplementary Table S2. The average, standard deviation and number of data points for the twisted plywood periodicity diameter. The corresponding $t$-values are $t_{0.05} = 2.00$ and $t_{exp} = 10.24$.

|            | Control bone | OI IV bone |
|------------|--------------|------------|
| $\bar{x}$ (µm) | 4.975        | 3.206      |
| $s$ (µm)   | 0.758        | 0.524      |
| $n$        | 30           | 24         |

Supplementary Table S3. The average, standard deviation and number of data points for the canaliculi diameter. The corresponding $t$-values are $t_{0.05} = 1.97$ and $t_{exp} = 7.61$.

|            | Control bone | OI IV bone |
|------------|--------------|------------|
| $\bar{x}$ (nm) | 523.2        | 420.4      |
| $s$ (nm)   | 143.0        | 115.8      |
| $n$        | 176          | 196        |

Appendix 3: Native Bone Pieces

Representative images of bone pieces used in this study and the coexistence of cortical and trabecular parts can be seen in Supplementary Figure S6. Cortical parts of the bone pieces were selected explicitly for further characterization methods as marked with the red rectangle. The black arrow indicates the direction of ion beam cutting during the Serial Slice and View experiment.

Supplementary Figure S6. Stereo microscope images of native bone pieces demonstrating the coexistence of cortical and trabecular parts.
Appendix 4: Representative optical images of OI and SEDC bone sections

Supplementary Figure S7. Representative OM images (a-i) of island-like structures as found in OI Type IV bone.
Supplementary Figure S8: Representative OM (a-g) and POM (h,i) images of SEDC bone, indicating the absence of island-like structures.
Appendix 5: Region of Interest for Serial Slice and View

Preserving the features of bone pieces is a critical aspect that needs careful attention. In order to make sure that the features are indeed preserved after sample preparation steps, such as fixation, staining, and cleaning, SEM imaging was conducted. As can be seen from Figure S9 twisted plywood motif, single collagen fibrils, and even characteristic D-spacing of collagen fibrils are observable.

**Supplementary Figure S9.** SEM images of control bone sample showing a) Haversian channel, twisted plywood motif and osteocytes, b) close-up image of twisted plywood motif, and c), d) collagen fibrils demonstrating the characteristic D-spacing.
Appendix 6: Nearest Neighbor Distance Analysis of Osteocytes

Supplementary Figure 10 shows representative POM images which were used to calculate the nearest neighbor distances of the osteocytes. After acquiring suitable images using POM, the images were loaded into MATLAB and Image J where osteocytes were marked manually.

Supplementary Figure S10. Optical microscope image of osteocyte network analysis of bone samples: a) osteocytes, b) osteocytes are marked to determine their positions, and c) Voronoi tessellation model showing the nearest neighbor network of osteocytes.
Appendix 7: Analysis of canaliculi diameters

Supplementary Figure S12 shows representative POM images demonstrating the canaliculi network marked with white arrows.

Supplementary Figure S11. Representative optical microscope images of the canaliculi network marked with white arrows. Black oval shapes indicate osteocytes.

Supplementary Figure S12. Representative images of canaliculi network from different serial slice and view experiments. Scale bars 1 µm.
Supplementary Figure S13. Several representative SEM images (a-l) used to calculate the periodicity of twisted plywood motif where regions that were excluded from our measurements are marked with red circles.
Appendix 9: Picrosirius Red Staining

Supplementary Figure S14. POM images of picrosirius red stained normal bone using two different orientations of the polarizer differing by 90° without a retardation plate (a,b) and with a retardation plate (c,d). An osteocyte (yellow) and Haversian canal (blue) are marked as a reference. Both samples show a change of color for certain structures depending on the orientation of the polarizer, making it difficult to draw any clear conclusion.

Appendix 10: Influence of bubble-like cavities in OI type IV bone on strength

Although bone during fracture is not behaving fully as an elastic material, we apply here linear elastic fracture mechanics (LEFM) to estimate the influence of the increased porosity due to the observed bubble-like cavities in OI IV bone on strength. For a brief introduction to LEFM, see, e.g., reference (20), or for a more extensive discussion, reference (21).

In LEFM the (fracture) strength \( S \) is estimated according

\[
S = \frac{1}{\xi} \left(\frac{2ER}{a}\right)^{1/2}
\]

where \( \xi \) is the so-called compliance factor (for surface defects approximately \( 1.12(\pi)^{1/2} \approx 2.0 \)), \( E \) the elastic modulus, \( R \) the fracture energy and \( a \) the defect size. As typically the defect size \( a \) in bone is about a 100 \( \mu m \) or more and the cavities are about 50 nm in size, we assume that
for $a$ and $\xi$ a similar value can be taken as for normal bone. Both $E$ and $R$ depend on the porosity $\phi$ for which various models for brittle materials are available. Useful empirical relations are [see e.g. reference \(^{(22,23)}\)]

$$\frac{E}{E_0} = \exp(-a\phi) \quad \text{and} \quad \frac{R}{R_0} = \exp(-b\phi)$$

Here $E_0$ and $R_0$ are the values for fully dense material. We consider them here to represent normal bone and thus also use $S_0$ to represent normal bone. Doing so, we should substitute $\Delta \phi$ due to the cavities for $\phi$. Evaluating leads to

$$\frac{S}{S_0} \cong \left[\exp(-a\Delta \phi) \exp(-b\Delta \phi)\right]^{1/2} = \exp\left[-\frac{(a+b)}{2}\right] \Delta \phi$$

The values for $a$ and $b$ reported in the literature scatter considerably, typical values being $a \approx 4.0$ and $b \approx 4.0^{(22)}$, so that $\exp[-(a+b)/2] = \exp(-4.0) \approx 0.0183$.

The value for $\Delta \phi$ for OI IV bone was estimated as the area fraction of the cavities as measured from 4 different images from the SSV procedure which were at least 2 $\mu$m apart from each other (Supplementary Figure 15). In this way, the same cavities do not appear in different slices. The images were processed via ImageJ, first creating a binary image with a threshold depending on the intensity values of the image used. Thereafter the area fractions of the binary images were measured, resulting in 2.92%, 2.68%, 2.88%, and 2.47%. The average value is $\Delta \phi = 0.0274 \pm 0.0021$, where $\pm$ indicates the sample standard deviation.

Consequently, for $S/S_0$ we obtain $S/S_0 = \exp[-(a+b)/2]\Delta \phi/S_0 = 0.0183^{0.0274} = 0.90$. Although the model for the porosity dependence used is primarily chosen for ease of use, other models as described in ref \(^{(22,23)}\) yield similar values. Also varying the exponents $a$ and $b$ down does not essentially change the result: for $a = b = 3$, $S/S_0 = 0.0498^{0.0274} = 0.92$ and for $a = b = 2$, $S/S_0 = 0.135^{0.0274} = 0.95$. At any rate, the effect of the cavities on stress concentration is neglected, which, if done, will increase $\xi$ and thus this strength decrease estimate is an
approximate lower limit. Moreover, adding the increase in porosity due to the caniculi,
\( \Delta \phi(\text{caniculi}) = 0.0074 \), we obtain
\( \Delta \phi = \Delta \phi(\text{bubble}) + \Delta \phi(\text{caniculi}) = 0.0345 \), and leading to
\( S/S_0 = 0.0183^{0.0345} = 0.87 \) for \( a = b = 4 \) and \( S/S_0 = 0.0498^{0.0345} = 0.90 \) for \( a = b = 3 \). However, the
strength of normal bone shows considerable scatter (see, e.g., ref. \(^{24-26}\)) and the decrease
estimated falls within the scatter observed. Summarizing, although the result \( S/S_0 \approx 0.90 \)
implies a significant decrease in strength, we conclude that this decrease is insufficient to
explain the rather fragile behavior of OI IV bone.

**Supplementary Figure S15.** Representative SEM images from SSV showing: a) Cross-sections of bubble-like
cavities (marked with white arrows), b) Determining the number of bubble-like cavities observed in the slice
desired result from SSV resulted in counting 106 cavities, c) Thresholding the image by marking the black dots
automatically depending on the intensity values which indicates where the cavities are located and d) The result
obtained from area fraction calculation by using Image J software.
Appendix 11: Periodicity of Twisted Plywood Motif

| Bone Type   | Thickness of Twisted Plywood Motif (µm) |
|-------------|----------------------------------------|
| OI Type IV  | 3.2 ± 0.52                              |
| Control     | 4.9 ± 0.75                              |

**Supplementary Table S4.** The average thickness of twisted plywood motif for each bone type. The ± indicates the sample standard deviation using approximately 200 measurements.

Appendix 12: Overview of the TEM lamella from control and OI type IV bone

Supplementary Figure S16 shows an overview of the control and OI type IV human bone lamella. It can be seen that most of the area shows clear banding patterns that are used to calculate the difference between control and OI type IV bone. The areas in the yellow boxes are some of the example regions where a clear D-spacing is visible.

**Supplementary Figure S16.** 2D projection of tomographic reconstruction slices (20 z-slices averaged) of a) SEDC and b) OI Type IV bone. Scale bars 200 nm.
Supplementary Figure S17. 2D projection images of (a-d) control bone and (i-iv) profiles of the areas marked by the blue rectangles in (a-d) showing the ~67 nm periodicity of the collagen fibril.
Supplementary Figure S18. 2D projection images of (a-d) OI Type IV bone and (i-iv) profiles of the areas marked by the blue rectangles in (a-d) showing the smaller periodicity of the collagen fibril compared with control counterpart.
Appendix 13: Analysis of intrafibrillar crystal orientations

To determine the orientation of the intrafibrillar crystals, an orientation analysis was performed. Tomographic slices were loaded as separate images to Matlab and the long and short axis of the crystals were manually set by clicking the four edges of the crystals. In order to avoid measuring the same crystals, the slices from the reconstruction were picked in a way that they are at least 50 nm (in z-direction) and 10 nm (in y-direction) apart from each other.

Supplementary Figure S19. Orientation analysis of the tomographic reconstruction slices in which the length of long (yellow) and short (red) axes of the crystals are analyzed. (a) A representative image of mineralized collagen fibril in control human bone viewed from the top and (b,c) along the collagen fibril, respectively. (d) A representative image of mineralized collagen fibril in OI type IV bone viewed from the top and (e,f) along the collagen fibril, respectively.

Furthermore, in order to determine the full width at half maxima in control and OI type IV bone a Gaussian fitting was used to the results obtained from orientation analysis from the
intrafibrillar crystals (Figure 12 and Figure 13 in the main text). Full width at half maximum values can be seen in Supplementary Figure S20.

Supplementary Figure S20. The experimentally determined distribution of intrafibrillar crystals in longitudinal orientations and the fitted Gaussian distribution (blue line) for a) SEDC bone and b) OI type IV bone.

Appendix 14: Supplementary Movies

Supplementary Movie S1. Stack of images from SSV procedure showing different types of motifs in control bone.

Supplementary Movie S2. Stack of images from SSV procedure showing different types of motifs in OI Type IV bone.

Supplementary Movie S3. Stack of images from SSV procedure showing bubble-like small cavities found in OI Type IV bone from the embedded sample.

Supplementary Movie S4. Stack of images from SSV procedure showing bubble-like small cavities found in OI Type IV bone in the air-dried sample.

Supplementary Movie S5. Electron tomographic reconstruction slices viewed from the top of the SEDC bone lamella, showing an overview of the sample.

Supplementary Movie S6. Electron tomographic reconstruction slices viewed from the top of the OI Type IV bone lamella, showing an overview of the sample.

Supplementary Movie S7. Electron tomographic reconstruction slices viewed from the top of the single collagen fibril in OI Type IV bone.

Supplementary Movie S8. Electron tomographic reconstruction slices viewed along the single collagen fibril in OI Type IV bone.
REFERENCES

1. Sambrook J, Maniatis T, Fritsch EF. Molecular cloning: A laboratory manual, 2nd Edition. 2nd ed. Spring Harb. Lab. Press. New York; 1989.

2. Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K. Chemical and physical basics of routine formaldehyde fixation. J. Oral Maxillofac. Pathol. 2012;16(3):400–5.

3. Bedino JH. Embalming Chemistry: Glutaraldehyde versus Formaldehyde. Expand. Encycl. Mortu. Pract. 2003;(649):2614–32.

4. Kiernan JA. Formaldehyde, Formalin, Paraformaldehyde And Glutaraldehyde: What They Are And What They Do. Micros. Today. 2000;8(1):8–13.

5. Veis A, Cohen J. The Degradation of Collagen. II. The Solubilization Process in the Acid pH Range. J. Am. Chem. Soc. 1955;77(9):2364–8.

6. Cury JA, Bragotto C, Valdrighi L. The demineralizing efficiency of EDTA solutions on dentin. Oral Surgery, Oral Med. Oral Pathol. 2005;52(4):446–8.

7. Pérez VC, Cárdenas MEM, Planells US. The possible role of pH changes during EDTA demineralization of teeth. Oral Surgery, Oral Med. Oral Pathol. 1989;68(2):220–2.

8. Echlin P. Handbook of Sample Preparation for Scanning Electron Microscopy and X-Ray Microanalysis. Handb. Sample Prep. Scanning Electron Microsc. X-Ray Microanal. 2009.

9. Kelley RO, Dekker RAF, Bluemink JG. Ligand-mediated osmium binding: Its application in coating biological specimens for scanning electron microscopy. J. Ultrasructure Res. 1973;45(3–4):254–8.

10. Golding CG, Lamboo LL, Beniac DR, Booth TF. The scanning electron microscope in microbiology and diagnosis of infectious disease. Sci. Rep. Nature Publishing Group; 2016;6(1):26516.

11. Seligman AM, Wasserkrug HL, Hanker JS. A new staining method (OTO) for enhancing contrast of lipid-containing membranes and droplets in osmium tetroxide--fixed tissue with osmiophilic thiocarbohydrazide(TCH). J. Cell Biol. 1966;30(2):424–32.

12. White DL, Andrews SB, Faller JW, Barnett RJ. The chemical nature of osmium tetroxide fixation and staining of membranes by X-ray photoelectron spectroscopy. BBA - Biomembr. 1976;436(3):577–92.

13. Gupta S, Aggarwal R, Gupta V, Vij R, Tyagi N, Misra A. Picrosirius red: a better polarizing stain. J. Histotechnol. Taylor & Francis; 2017;40(2):46–53.

14. Kulkarni RR, Sarvade SD, Boaz K, Srikant N, Nandita KP, Jlewis A. Polarizing and light microscopic analysis of mineralized components and stromal elements in fibrous ossifying lesions. J. Clin. Diagnostic Res. 2014;8(6):42–5.

15. Dayan D, Hiss Y, Hirshberg A, Bubis JJ, Wolman M. Are the polarization colors of Picrosirius red-stained collagen determined only by the diameter of the fibers? Histochemistry. 1989;93(1):27–9.
16. Newman GR, Hobot JA. Resins for combined light and electron microscopy: A half century of development. Histochem. J. 1999;31(8):495–505.

17. Kushida H. On an Epoxy Resin Embedding Method for Ultra-thin Sectioning. Electron Microscopy. 1959;8(1):72–5.

18. Finck H. Epoxy resins in electron microscopy. J. Biophys. Biochem. Cytol. 1960;7:27–30.

19. Luft JD. Improvements Embedding in Epoxy Resin Methods. J Biophys Biochem Cytol. 1961;9:409–14.

20. With G de. Polymer Coatings: Guide to Chemistry, Characterization and Selected Applications. Polym. Coatings. 2019.

21. With G De. Microengineering of Metals and Ceramics Nanomaterials by Severe Plastic Deformation Handbook of Ceramic Hard Materials. 2006.

22. Rice RW. Microstructure Dependence of Mechanical Behavior of Ceramics [Internet]. Prop. Microstruct. Treatise Mater. Sci. Technol. 1977.

23. Rice RW. Porosity of Ceramics. Mater. Sci. Eng. A. 2000;280(2):358–9.

24. Zioupos P, Currey JD. Changes in the stiffness, strength, and toughness of human cortical bone with age. Bone. 1998;22(1):57–66.

25. Wagoner Johnson AJ, Herschler BA. A review of the mechanical behavior of CaP and CaP/polymer composites for applications in bone replacement and repair. Acta Biomater. 2011;7(1):16–30.

26. Gomes DS, Santos AMC, Neves GA, Menezes RR. A brief review on hydroxyapatite production and use in biomedicine. Ceramica. 2019;65(374):282–302.