Bone Material Quality

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What You Will Learn in This Chapter

One important role of our skeleton is to fulfil mechanical functions, in particular the mechanical support of our body enabling complex movement and locomotion as well as safety housing for sensitive organs. In this book chapter, you will learn about the characteristics of the bone material and their role for the mechanical performance of the bone at organ level. First, we give an overview of the hierarchical organization of the bone, the heterogeneity of bone material structure and the composition from microscale to nanoscale. Second, we describe important technical terms related to bone deformation; we present bone material quality parameters and discuss their role in bone deformation. Third, we briefly explain the most important current techniques for the characterization of the bone material quality such as light and electron microscopy methods, X-ray scattering and fluorescence, vibrational spectroscopic techniques, etc. Forth, we present material characteristics in clinical examples of bone fragility including postmenopausal osteoporosis and treatment and osteogenesis imperfecta.

1.1   Structure and Composition of Bone at Material Level

Bone is a *lightweight structured* organ, i.e. it is constructed following the principle to have maximum resistance to fracture based on as little material/mass as possible. For this purpose, bone is hierarchically organized [1]. One may distinguish between organ, tissue and material levels (◘ Fig. 1.1). The *organ level* (outer geometry, shape and dimensions of the whole bone) and the *tissue level* (inner architecture of the bone) and their role in bone fragility are described in the book ▶ Chap. 6 of Pietschmann et al. In general, all hierarchical levels are optimized for the excellent mechanical competence of the bone [2]. As discussed later, bone diseases associated with fragility can be caused by either affecting single or multiple hierarchical levels of bone organization.

1.1.1   Bone Structural Units (BSUs)

The ongoing process of bone resorption and formation (bone remodelling) is responsible for a remarkable heterogeneity of the bone material. During bone remodelling, bone formation is occurring on surfaces which were generated by previous resorption due to osteoclastic activity (see book ▶ Chap. 3 of Teti and Rucci) and covered by a thin non-collagenous protein cement layer. The bone material which is deposited on a pre-existing bone surface by the coordinated action of numerous osteoblasts (see book ▶ Chap. 2 of Fratzl-Zelman and Varga) during formation time is called a bone structural unit (BSU) (in trabecular bone also termed «bone packet» or «hemiosteon», in cortical bone «osteon»). Depending on the time passed from formation, each BSU has its specific tissue age. The bone turnover rate (frequency of remodelling cycles) determines the distribution and the average of these BSUs' tissue ages.

An important feature of a BSU is its mineral content, the bone matrix mineralization density. When bone matrix is deposited by the osteoblasts, its mineral accumulation follows a specific time course. First, during the osteoid phase, the collagenous matrix is maturing without being mineralized. Second, after onset of mineralization, mineral accumulates rapidly during the primary mineralization. This is followed by a third period with slowly increasing matrix mineralization (secondary mineralization) which takes
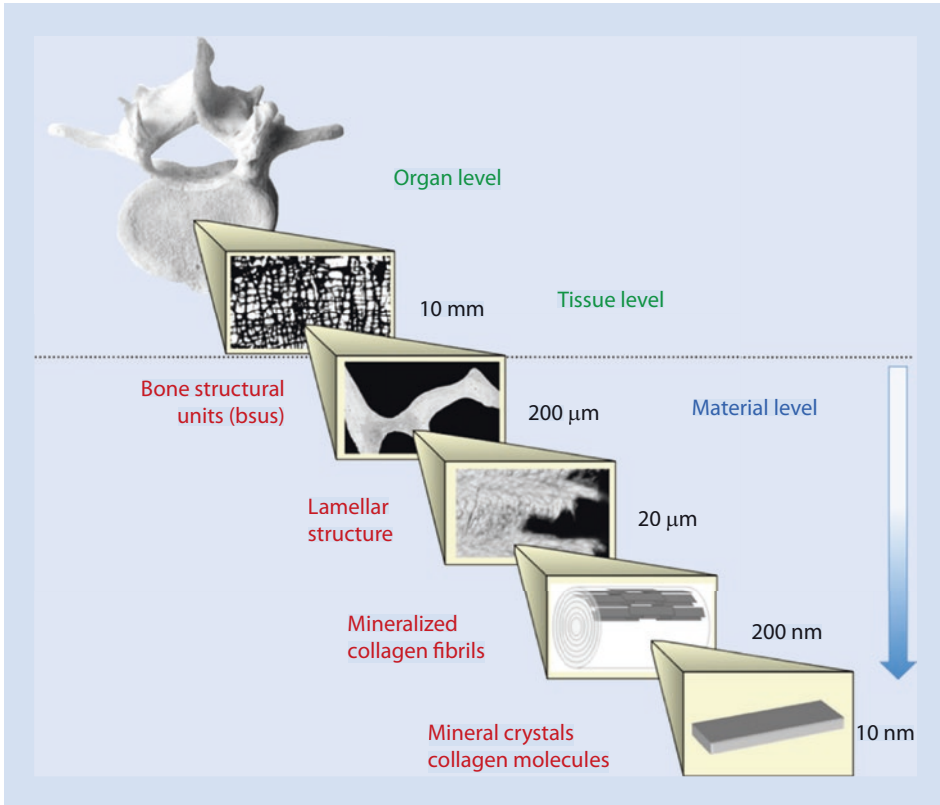
Fig. 1.1 Hierarchical structure of the bone

months to years until the plateau of full mineralization is achieved [3, 4]. Due to the time course of these processes, the bone is composed predominantly of BSUs in the secondary mineralization phase. In healthy individuals, the global mineralization pattern reflects the tissue ages of the BSUs (younger bone tissue is less, older higher mineralized) (■ Fig. 1.2).

Another characteristic feature of a BSU is its lamellar collagen fibril arrangement (the next lower level of bone material organization; see ▶ Sect. 1.2). In trabecular bone a uniform lamellar structure parallel to the trabecular surface is formed, while in cortical bone, the lamellar bone is arranged cylindrically around a Haversian canal (a canal for blood vessels, nerves and bone marrow). In general, this orientation is abruptly changing at the cement line, which represents the demarcation to the adjacent BSU (■ Fig. 1.3).

When the osteoblasts deposit the matrix, part of these cells gets entrapped in the mineralized bone matrix and differentiates into osteocytes which form a dense osteocyte lacunar-canalicular network [5] with a density of about 20,000 osteocyte lacunae per mm^3 [6] and an average osteonal canalicular density of $1.6 \pm 0.8 \times 10^6/mm^3$ [7]. There is evidence that this network is involved in mechanosensing of load by local bone deformation and in cell communication. Moreover, this lacuno-canalicular system provides an enormous inner bone surface of about 215 m^2 [8] (see book ▶ Chap. 2 of Fratzl-Zelman and Varga), which might be used to regulate rapidly the homeostasis of elements like Ca, P and Mg.
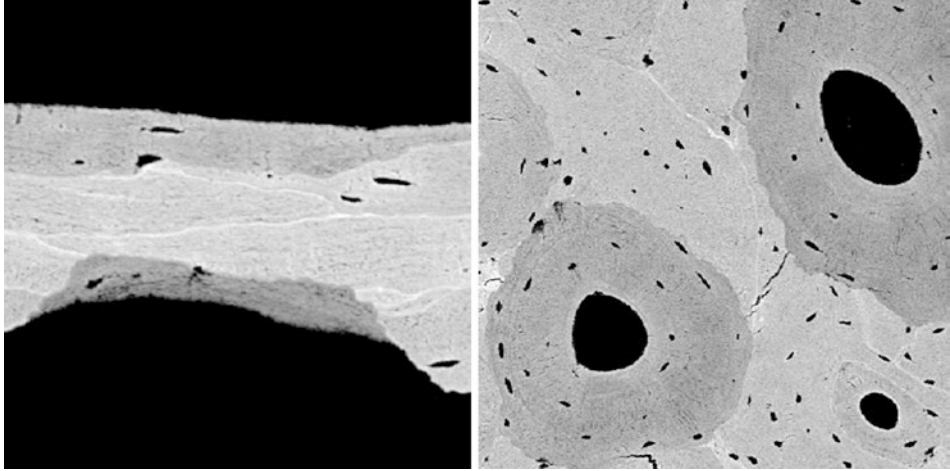
Fig. 1.2 Backscattered electron images of BSUs. Younger BSUs with lower mineral content appear dark, older with higher mineral content bright; *left*, BSUs in trabecular bone (bone packets); *right*, BSUs in cortical bone (osteons)

Fig. 1.3 Lamellar collagen fibril arrangement based on polarized light microscopy of a thin section of trabecular bone
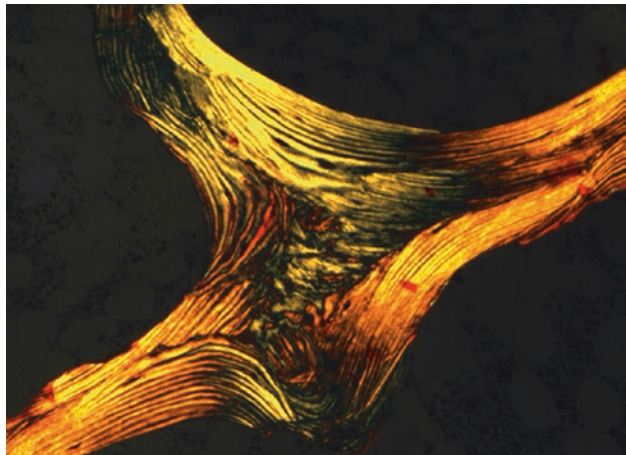
1.1.2 Lamellar Arrangement of Collagen Fibrils

In mature bone, the collagen fibrils are arranged in parallel bundles, which are embedded in an extra-fibrillar matrix (organic and mineral) [9]. They are further organized to form a lamellar structure with a period of about 7 μm produced by a periodic change of the dominant fibril orientation (this is in contrast to primary/callus bone, where the fibrils are oriented randomly) [10]. A narrow and a wide lamella can be distinguished. In osteonal bone, a helicoidal structure of the collagen fibrils arranged in a spiral around the blood vessel can be observed [10].
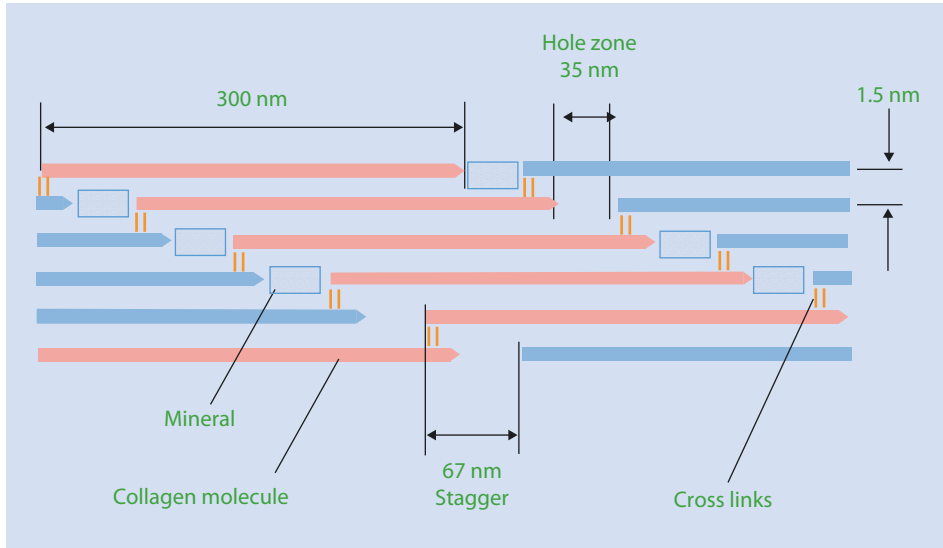
◘ Fig. 1.4 Schematic drawing of collagen fibril with starting mineralization. The colours of the collagen fibrils indicate the periodicity in their arrangement

1.1.3 Mineralized Collagen Fibrils

The mineralized collagen fibril is the basic building block of the bone material [11]. Within the fibril, about 300 nm long molecules line up parallel staggered and form a characteristic banded structure with a 67 nm periodicity of hole and overlap zones of collagen molecules [12] (◘ Fig. 1.4). It is assumed that during the mineralization process (see ▶ Sect. 1.1), the mineral crystals start growing predominantly in the hole zones of the fibril to form plate-like mineral particles of 1.5 to 4.5 nm thickness, about 50 nm length and 25 nm width [13, 14]. They are also arranged in a parallel, staggered manner with orientation to the longitudinal axis of the fibrils [2].

1.1.4 Collagen and Mineral

The components of the nanocomposite bone material are *organic molecules* and *mineral crystals*. The main organic part (90%) is collagen type I, and the rest (10%) are non-collagenous proteins which are both important for initiation of mineralization [15, 16]. After the secretion of procollagen molecules by the osteoblasts, the collagen fibrils form by self-assembly and get mineralized later (see ▶ Sect. 1.1). Enzymatic and non-enzymatic cross-links are formed between the collagen helices to stabilize the collagen fibrils. Divalent cross-links are first formed by enzymatic involvement (lysyl oxidase) partly followed by transformation to trivalent cross-links spontaneously [2].

The mineral phase of the bone is calcium phosphate salt in the form of nanosized crystals of hydroxyapatite [17]. In the bone, these hydroxyapatite minerals ($Ca_5(PO_4)_3OH$) are not pure but contain other elements like F, Na, K, Mg and Sr. Commonly, some phosphate or hydroxyl groups are replaced by carbonate groups (type B carbonate substitution) and may perturb crystal lattice perfection [18].

1.2   Quality and Mechanical Properties of Bone Material

In our skeletal system, the bones are exposed to various loading conditions. How much of these loads are tolerated without occurrence of a fracture is not only dependent on the bone quantity/mass but also on its quality at all hierarchical structural levels.

1.2.1   From Mechanical Properties of Whole Bone to Properties of Bone Material

The bones of our skeletal system can experience three principle loading modes: tension, compression and shear and the combination thereof. Estimation of whole-bone mechanical properties is typically based on compression tests (e.g. for vertebrae) or three- and four-point bending tests (for long bones); for details see reference [19]. During a typical compression test, e.g. both the progress of loading (increase of force) with time and the corresponding deformation (displacement of the vertebral endplate) are recorded in the load-displacement curve. Characteristic mechanical indices derived from this curve (◘ Fig. 1.5) are the applied load (*y*-axis), the resulting displacement (*x*-axis), the linear part of the curve (the elastic region), the slope of the elastic region (stiffness), the non-linear region (indicating the plastic region, where deformation is irreversible), the displacement and load (failure load) at which bone fractures and the entire area under the curve which represents the work to failure. Furthermore, single loading (increasing load), cyclic loading (repetitive lower magnitude loading) or creep experiments (constant load) can be distinguished but will not be further described here.
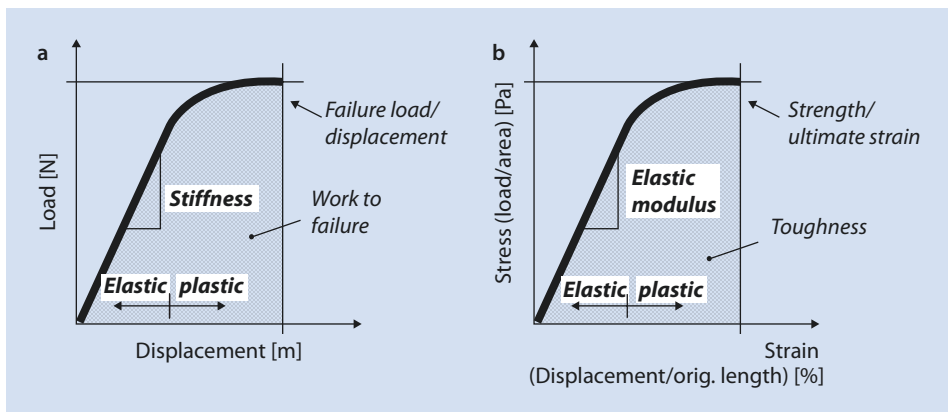
◘ **Fig. 1.5**   Characteristic mechanical indices derived from **a** load-displacement or **b** stress-strain curve

Such mechanical tests on whole bone or even on small tissue samples are dependent on both geometrical/structural and material properties. For instance, an important geometrical parameter of the long bone is its cross-sectional outer radius, as the bending stiffness of the bone increases with a power 4 law of its outer radius. Moreover, cortical thickness, porosity, density and size of Haversian canals as well as trabecular architecture (see also book ▶ Chap. 6 of Pietschmann et al.) play an important role for whole-bone mechanical properties. In general, if sufficient geometrical information is available, *indirect estimates* of mechanical properties of material (in contrast to the *direct measures* in ▶ Sect. 1.3, where an overview of methods for bone material characterization is given) can be deduced from such experiments. Subsequently, the load-displacement curves normalized for bone/sample geometry reveal indirect estimates of bone material such as stress, strain, E-modulus, strength and toughness (◘ Fig. 1.5).

1.2.2 Bone Material Quality

Apart from macroscopic changes, deformation and failure at multiple lower size scales (at material level) play a role in the deformation of the bone as a whole.

Role of Mineralization Pattern for Deformation

The slope of the pre-yield (elastic) region in the stress-strain curve was shown to be related to the degree of matrix mineralization [20]. The higher the degree of mineralization or density of the mineral particles, the larger is the slope (the stiffer is the material) [21]. Both hypo- and hypermineralization may compromise the mechanical performance of the bone [22]. Our previous findings suggest an optimal bone mineralization density distribution throughout the bone matrix [23]. Another important parameter of mineralization is its heterogeneity (introduced by the BSUs) that may play a role for crack propagation and deflection as discussed below.

Role of the Microcomposite for Deformation

The combination of soft and tough collagen with the hard and brittle mineral leads to an extraordinary composite which is stiff but not brittle. The special arrangement and the quality of the two components are essential as tensile stress on the bone material is transmitted through the collagenous matrix by shear to the crystals protecting them from fracture [9, 24]. Abnormal tendon collagen in a mouse model of osteogenesis imperfecta was found to have decreased yield and ultimate stress and strain as well as decreased toughness [25]. Abnormal enzymatic and non-enzymatic cross-linking was related to increased bone fragility [26–30]. Additionally, deviations from normal mineral crystal shape and size as observed after sodium fluoride treatment were linked to decreased bone material strength [31]; see ▶ Sect. 4.1.

For the direct observation of *deformation at nanoscale*, sophisticated techniques including in situ experiments at the synchrotron are required which enable simultaneous mechanical testing and high-resolution imaging. Approximately 58% of the tissue strain in the elastic region (overall deformation) during tensile testing occurs via shearing within the extra-fibrillar matrix (between the fibrils) and 42% within the mineralized fibrils. The mineral platelets receive only 16% of the overall deformation. This indicates a natural stress shielding design where the brittle mineral particles see less strain to avoid catastrophic failure. When deformation in these tensile tests exceeded the elastic region, fibrils showed no further elongation suggesting the slippage of the fibrils [9].

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■ **Fig. 1.6** Backscattered electron image of crack propagation perpendicular to the lamellar orientation of the bone material. «Zig-zag» line indicates the requirement of much energy for propagation
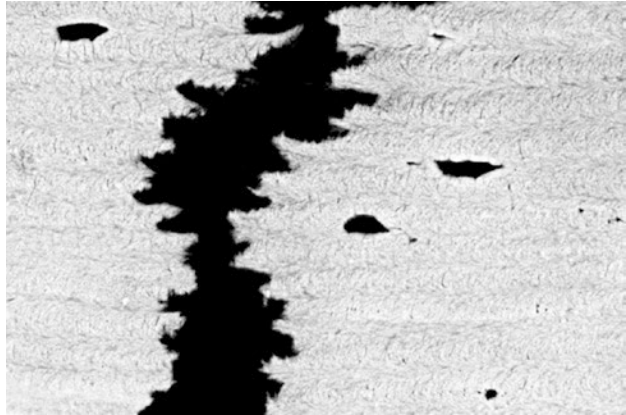
Toughening Mechanisms of Bone Material

When the bone is loaded beyond the elastic region, damage and cracks (deformation) become apparent at *microscopic scale*. It was observed that the formation of such microcracks and/or diffuse microdamage increased bone toughness, thus being a mechanism of energy absorption. The underlying mechanisms were proposed to be (i) crack (or «ligament») bridging and (ii) crack deflection [32]. Indeed, lamellar orientation plays an important role, and it was reported that it requires much higher energies to propagate a crack perpendicular than parallel to the lamellar orientation [33] (■ Fig. 1.6). Disturbed lamellar orientation was linked to bone fragility in pycnodysostosis [34]. Heterogeneity of bone material in general (interfaces with changing mineral content, lamellar orientation, organic matrix) might be important for crack deviation and crack stopping [35–38]. There is also evidence that the osteocyte lacunar-canalicular network plays a role in crack initiation and propagation [7].

Although the formation of microcracks might enhance toughness, once they have been formed, their presence might weaken the bone material in response to future external loading [39]. Removal of microdamage has been proposed to be essential and to be conducted by targeted resorption. Microdamage involves the damage of the osteocytic network, and signals from the osteocytes have been proposed to attract the osteoclasts to these damaged sites for resorption and removal of damaged bone material. This targeted bone resorption is generally assumed to be an important bone remodelling mechanism.

1.3   Overview of Methods for Characterization of Bone Material

During recent years, several methods and the combination thereof have been developed which allow comprehensive characterization of structural and mechanical properties at high spatial resolution. We give here an overview of selected methods and their application on bone material; for more information, see reference [40].

■ **Arrangement and Composition of Organic Matrix**
Polarized light microscopy: The lamellar arrangement of the collagen fibrils can be visualized due to the birefringence of collagen.

Fourier transform infrared (FTIR) and Raman microspectroscopy (RM): Vibrational spectroscopic methods are sensitive to bond vibrations thus providing information on the functional groups present in the mineral and organic matrix components, as well as the short- and medium-range interactions between them («molecular neighbourhood»). In FTIR thin (~4 μm) sections are required, whereas in RM, sections and/or bone blocks are analysed. Both techniques allow the characterization of the relative amounts of organic matrix and mineral, information on carbonate substitution in the mineral, mineral crystallinity/maturation and collagen cross-linking. RM has been recently established also for the measurement of lipids, proteoglycans and advanced glycation end products (AGEs) [41, 42]. Recently, a new parameter has been introduced which provides information on the nanoporosity (a surrogate for tissue water) of bone sample. During sample preparation, the embedding substrate penetrates the porosities of the bone material which had been previously (in-vivo) filled with water. In this way, the relative amount of the embedding material can be used for an estimation of the nanoporosity/water content within the bone material [43].

■ Quantification of Mineral Content Distribution

Quantitative Backscattered Electron Imaging (qBEI): qBEI in the scanning electron microscope is a sensitive and frequently applied technique for the analysis of the mineralization pattern [23]. It requires block samples of undecalcified polymethylmethacrylate embedded tissue with a flat surface. qBEI is based on the dependency of the backscattered electron intensity on the local atomic number in the sample (mainly dependent on the local calcium concentration). The higher the calcium concentration, the higher the backscattered electron intensities and the brighter are the pixel grey levels in the image. The images can be used for the analysis of histograms revealing the frequency of the different calcium concentrations (given in weight% Ca) in the studies bone sample (designated bone mineralization density distribution, BMDD), ▫ Fig. 1.7.

Quantitative Microradiography (qMR): This was the first method used to quantify the mineralization pattern. qMR is based on the unidirectional X-ray projection of 100 μm thick polymethylmethacrylate embedded bone sections and the measurement of the absorption of the former. The resulting microradiograph is analysed for its grey levels and grey level histograms having the meaning of mineral content in units of g/cm^3 [44].

Synchrotron Radiation Microtomography (SRμCT): SRμCT is the most modern method and provides 3-dimensional information on the mineral content in the bone sample. It is based on multidirectional projection and absorption of a focused, monochromatic X-ray beam by small bone samples (2 mm × 2 mm). The resulting data set allows 3-D reconstruction and mineral distribution measurement based on voxel grey levels with the meaning of mineral content in units of g/cm^3 [40].

■ Characteristics of Mineral Particles/Crystals

Scanning Small-Angle X-Ray Scattering (sSAXS): This method is based on the measurement of the scattered intensities very close to the incident beam direction (at small angles). In bone material analysis, this method is mainly used for information on the size distribution, the shape and the arrangement of the mineral particles of the nanocomposite material with size dimensions smaller than 50 nm. sSAXS is available at the laboratory or at the synchrotron (the latter allows to scan the sample with high spatial resolution of 3–10 μm; however, it requires very thin 3–10 μm bone sections) [14, 31]. Recently, sSAXS tomography was introduced which provides the 3D view of mineral particle properties of the bone [45].
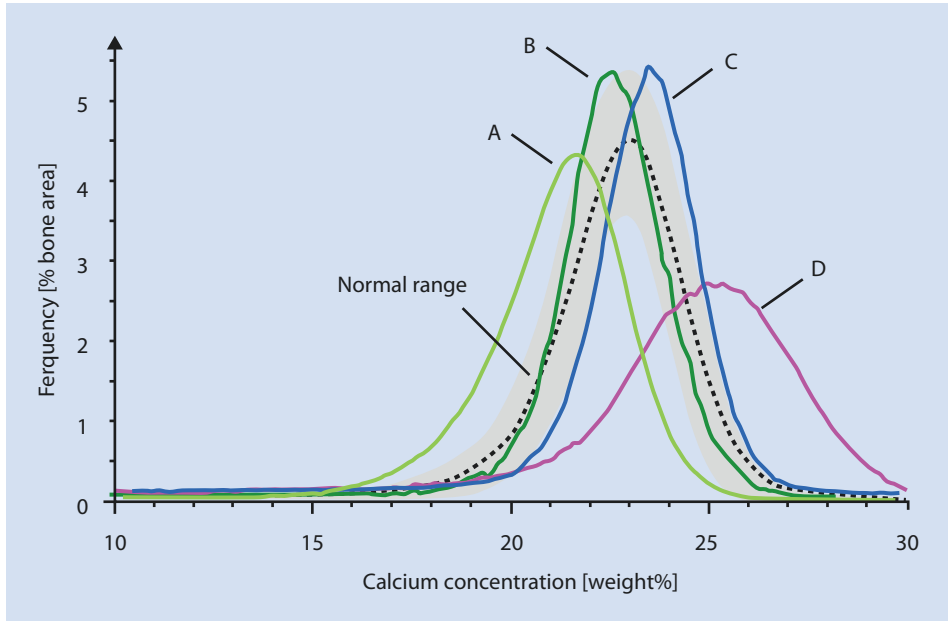
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◘ **Fig. 1.7**   Examples of bone mineralization density distribution in health and disease. *A* postmenopausal osteoporosis, *B* bisphosphonate treated, *C* osteogenesis imperfecta, *D* sodium fluoride treated bone

Scanning Wide-Angle X-Ray Scattering (sWAXS): The scattered X-ray intensities under larger (wide) angles can originate from the scattering/diffraction of the incident X-ray beam by the crystal lattice of the bone mineral particles and provide information on crystal lattice characteristics (lattice parameters of the crystal unit cell, crystallinity and crystal dimensions) [10].

Transmission Electron Microscopy (TEM): Another method to study the small bone mineral particles is TEM, which provides direct high-resolution images or might be used for electron microscopic tomography [40]. For TEM analysis, plastic embedded, ultra-thin bone sections (70–100 nm) are required. The energy of the electrons, which have to transmit the bone section, is in the range of 100–1000 keV. The image contrast is generated mainly by the difference in electron density of the structures in the sample. Single/isolated crystals in the viewing field can be analysed for their shape and dimensions.

■   **Elemental Composition**

X-Ray Fluorescence (ERF) Induced by Electrons or by X-Ray at the Synchrotron (SR-XRF): These techniques allow non-destructively, spatially resolved analysis of the elemental composition of the bone (Ca, P, Mg, Na, K). SR-XRF is applied when sufficient sensitivity (ppm concentration range) is needed, e.g. in the detection of trace elements (Pb, Sr, Mn, Zn, F) [46].

Visualization and Quantification of Osteocyte Lacunar-Canalicular Network (OLCN): The OLCN in the bone material can be studied in three dimensions by confocal laser scan-

ning microscopy (CLSM) after fluorescent staining of bulk bone samples with rhodamine G, e.g. which stains the organic phase of all inner surfaces of the OLCN [5]. Alternatively, synchrotron high-/nano-resolution X-ray computed tomography allows to visualize the OLCN without any staining but is limited to small bone volumes.

■ **Direct Measures of Mechanical Aspects of Bone Material**

In contrast to the indirect estimates of mechanical properties of bone material described above, there are methods available which result in a direct measure of bone material properties. These measures are independent of geometry and have the advantage that they can be obtained in a spatially resolved manner which is highly important in a heterogeneous material such as bone.

Scanning Acoustic Microscopy (SAM): The elastic properties of the sectioned bone sample can be visualized with spatial resolution up to 1 μm. SAM is based on generation and focusing of high-frequency acoustic waves to the bone followed by the detection of the echo signals [53]. The inherent contrast (amplitude or difference of time of flight in thin section) is caused by elastic interactions of the incident acoustic waves with the bone material, in particular with changes in lamellar orientation and local mineral content (◘ Fig. 1.8).

Nanoindentation: Direct mechanical measurements can be also obtained from nanoindentation at the atomic force microscope (AFM) [40]. In a controlled deformation experiment, the tip of the AFM (nanosized indenter) is pushed into the bone block or sectioned sample, and the load-displacement curve is recorded, from which the indentation modulus as well as the hardness can be derived. The latter outcomes were shown to generally correlate with local mineral content, despite quite large variation at a specific mineral content, and revealed a high anisotropy depending on the direction of the lamellar structure.
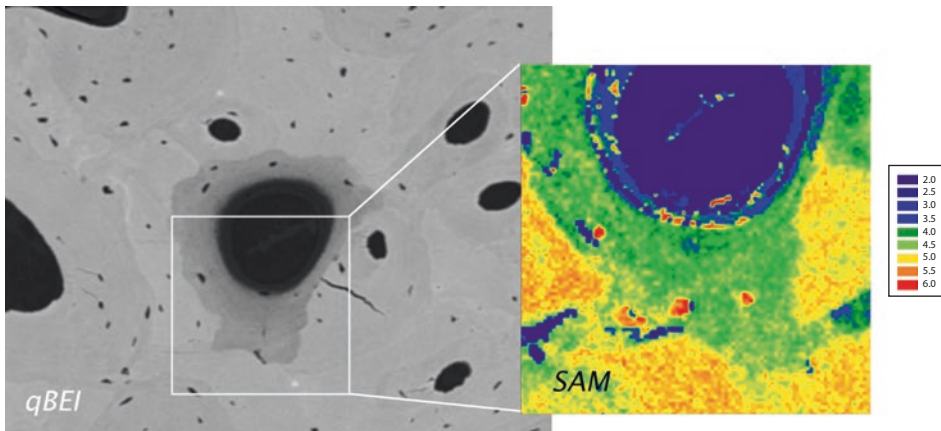
◘ **Fig. 1.8** Backscattered electron mapping of mineral content of osteonal bone (*left*), SAM velocity mapping of enlarged area (*right*). The higher the mineral content, the higher the ultrasound velocities. Both combined informations enable calculation of the local elastic modulus [53]

1.4   Bone Fragility Associated with Altered Bone Material

Commonly, the measurement of bone mineral density (BMD by DXA) is considered for fracture risk assessment and treatment decision for a patient. However, BMD alone is not a perfect predictor of fracture risk. With advancing development of techniques and with awareness of bone material quality being an important contributor to the whole-bone mechanical competence, the characterization/quantification of bone material properties has become an important tool of clinical application (differential diagnosis and treatment monitoring). Two examples are shown in the following.

1.4.1   Bone Material in Postmenopausal Osteoporosis and After Treatment

Postmenopausal osteoporosis is associated with low BMD due to bone loss and reduced bone matrix mineralization (shift of the BMDD to lower calcium concentrations in ◘ Fig. 1.7) as a consequence of increased bone turnover rates [47]. Additionally, altered cross-link patterns have been reported [48]. Commonly, postmenopausal osteoporosis is treated with anti-resorptive agents such as bisphosphonates. Interestingly, relatively small treatment-induced increases in BMD result in disproportionally higher fracture risk reduction of treated patients. The observed increase in degree of mineralization was suggested to be contributing to the improvement of bone quality during treatment [47].

Another strategy in osteoporosis therapy is anabolic treatment, for instance, with teriparatide or parathyroid hormone which are improving BMD by increasing bone volume while even transiently decreasing average bone matrix mineralization (due to large amounts of young BSUs). Another therapy to be mentioned in this context was the previous treatment with sodium fluoride. This treatment was not successful, as it had no positive effect on fracture risk reduction although it caused large increases in bone volume. Analysis of bone material formed under therapy revealed highly increased mineral content and an abnormal nanocomposite including increased size of the mineral particles [31].

1.4.2   Bone Material in Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a genetic disease associated with collagen fibril alterations and characterized by increased bone turnover and brittle bone phenotype [49]. The bone fragility is based on reduced tissue quality (reduced bone volume, abnormal architecture) and in particular on deviations from normal bone material quality. Decreased mechanical competence of collagen matrix and hypermineralization (shift of the BMDD to higher calcium concentrations in ◘ Fig. 1.7) has been linked to the increased brittleness in this disease [50]. Bisphosphonate treatment was found beneficial to patients with OI [49, 51]. The positive effect on fracture risk reduction in young patients with OI is based primarily on the increase in cortical width and cancellous bone volume [51] in these patients, while the bone material quality remains unaffected by treatment [52].

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