Why should we investigate bones at all?

Studies of Medicine do not put much focus on bones. Majority of those rare hours, during which medical students acquaint themselves with bones, are spent in Anatomy where only the macroscopic appearance of bones is presented. Despite a few hours on bone microscopic slides in Histology and Pathology, most of the students consider bones as something simple and dead, just an anchorage point for the muscles, not too different from a piece of stone or a wooden stick. The fact that bones can break does not change such an impression. Still, identifying and treating a bone fracture is probably one of the first and key associations to bones in general. It becomes more interesting when one checks statistics of bone fractures: there are actually two incidence peaks, one in youth and the other in senescence (1). Fractures in aged individuals commonly appear at the femoral neck (hip fractures) (2), with predilection for female sex (3) and occur due to a low-energy trauma (usually a fall from the standing height) (4). However, a fall is not sufficient to break the hip, considering that young bone would not break in such conditions. Hence, the main cause of easy bone fracturing must originate from the characteristics of the bone itself. So, if we really want to understand why bones do break, especially in elderly individuals, we have to reject common macroscopic perception of bone and try to understand what it really is.

Short overview of bone structure (bone hierarchical organization)

Bone is a very complex and hierarchically organized structure (5), which means that it has different organization and appearance at different length scales: at the macro-level, micro-level, and nano-level (figure 1). In simple terms, this actually means that the bone looks quite different depending on the scale of ob-
servation. Therefore, to profoundly understand what bone really is and to comprehend what determines bone strength, one has to consider bone features at various hierarchical levels.

First, macroscopic observation shows the shape and the size of the bone. It is frequently visible with a naked eye after making a cross-section that bones consist of cortical and trabecular compartments (figure 1B-D). Cortical (or compact) bone is the outer bony layer with a very low porosity (6), in contrast to the porous trabecular (cancellous or spongy) compartment consisting of a network of interconnected bony plates or rods (trabeculae) that fills the bone interior (5, 7) (figure 1B-C).

Going down to the microscopic level, it exposes bone as a living tissue composed of cells with specific functions (bone-forming osteoblasts, bone-resorbing osteoclasts, and the most numerous osteocytes) (7). These cells are active, extensively interconnected and intensively communicating to maintain or adapt bone structure to the local mechanical and global metabolic needs of the organism (8-10). Most of the bone volume is occupied by extracellular matrix (bone material or bone matrix).

Table 1. Main properties of the bone mineral component

<table>
<thead>
<tr>
<th>Mineral characteristic</th>
<th>Parameter</th>
<th>Method of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree/distribution of mineralization</td>
<td>Calcium weight-percentage</td>
<td>Quantitative backscatter electron imaging</td>
</tr>
<tr>
<td>Degree of carbonate substitution</td>
<td>Carbonate-to-phosphate ratio</td>
<td>Raman, FTIR</td>
</tr>
<tr>
<td>Type of hydroxyapatite</td>
<td>Calcium-to-phosphorus ratio</td>
<td>EDS</td>
</tr>
<tr>
<td>Morphological characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape and size of mineral crystals</td>
<td>Crystal length</td>
<td>SEM, AFM, XRD</td>
</tr>
<tr>
<td>Crystal perfection</td>
<td>Crystallinity</td>
<td>XRD, Raman, FTIR</td>
</tr>
<tr>
<td>Roughness</td>
<td>Surface roughness</td>
<td>AFM-PSD</td>
</tr>
<tr>
<td>Structural complexity</td>
<td>Fractal roughness</td>
<td>AFM-PSD</td>
</tr>
</tbody>
</table>

Going further down to the nano-level (bone material or matrix level) reveals that bone matrix is a nano-composite material (figure 1E-F) composed of mineral crystals, organic phase (mostly collagen fibrils), and water (5, 11).

Nanostructure of bone

Bone mineral is carbonated hydroxyapatite; however, its exact physicochemical characteristics are complex and still attract research attention (12-13). Bone mineral is organized in particles of various sizes (commonly termed “mineral crystals” irrespective of their true degree of crystallinity), mostly of plate-like shape (13). Collagen type I – the main constituent of the organic phase (90%) (5-6) – is organized in fibrils that are reinforced by mineral crystals (figure 1E-F). The remaining 10% of organic phase are non-collagenous proteins that provide attachment to fibrils, crystals and cells, and contribute to bone toughness (5, 11, 14).

It is believed that the mineral part mainly determines bone mechanical properties, especially hardness and strength of bone (15-16). A number of chemical and morphological properties can be analyzed to describe bone mineral component (table 1). Clearly, understanding of bone at nano-level could not be possible without the use of advanced technology (table 1) (9, 17-19). Atomic force microscopy (AFM) is another powerful tool for characterization of nanomaterials that has been recently applied to bones (11, 20). AFM allows great spatial resolution without the need of excessive sample preparation. In contrast to light or electron microscopy that use light or electron beams and system of lenses to obtain the image of the specimen, the AFM uses a sharp mechanical probe to physically “touch” the specimen and provide a 3D image of the specimen’s surface topography (figure 2). Moreover, AFM can distinguish between the areas of different material properties and allow mechanical characterization of materials in addition to imaging (AFM nanoindentation) (11, 21).

First AFM studies were mainly qualitative and advanced the knowledge on bone nanostructure. For instance, applying AFM on bovine vertebral trabeculae showed that interfibrillar mineral crystals are not of uniform shape and size in the same bone (22). Analysis of the outer surface of human trabecular bone showed mainly bare collagen fibrils, while fracture surface of bovine trabecula exposed mineralized collagen fibrils (23). The mineralized fibrils detected on fracture surfaces led to the assumption that the mineral-to-mineral interface

![Figure 2. Cortical bone nanostructure at the femoral neck of an elderly woman without bone fracture (A,B) and with bone fracture (C,D): AFM Topography (A,C) and corresponding Phase images (B,D); Scale bar = 200 nm.](image-url)
is the weakest link in bone and that fractures are mostly initiated there (23). AFM studies in animal bone elegantly showed collagen fibrils after removal of the mineral particles by means of EDTA or NaF (24-26). Conversely, treatment with collagenase allowed better visualization of bone minerals revealing that 70% of mineral is placed around collagen fibrils (interfibrillar or extrafibrillar mineral) (26), while the rest is located inside the collagen fibrils (15, 26).

Recent AFM studies made a step forward by introducing quantitative analysis of bone nanostructure and proved AFM as a powerful tool for the assessment of age-related effects on the bone mineral (21, 27-30). Namely, our AFM study of the trabecular bone at the femoral neck revealed an increased size of mineral crystals in aged, compared to young women (30). As a rule of thumb in materials science, structures composed of larger particles have a decreased material strength (31); hence, these nano-structural differences (30) contribute to increased fragility of the femoral neck in aged females. Crystal size increases generally with aging, but not at the same rate in all individuals. The external cortical surface of the femoral neck in postmenopausal women who sustained a hip fracture displayed larger mineral crystals than in age-matched women without skeletal diseases (27) (Figure 2). Apart from increased crystal size, the femoral neck cortex in the fracture group, showed higher degree of mineralization (27), another factor leading to increased brittleness and impaired resistance to fracture.

Application of Fourier-transform based Power Spectral Density analysis (PSD) – another novel methodological approach with AFM – proved useful for explaining differential trabecular bone fragility across the age at the nanometer scale (29). Namely, decreased fractal dimension of the interfibrillar mineral of the femoral neck trabeculae in the elderly denoted their reduced structural complexity and surface roughness. These findings suggest a decreased ability to dissipate energy during loading, which in turn leads to increased brittleness and bone fragility in aged persons (29).

Finally, recent nano-scale mechanical assessment of the femoral neck trabeculae, in young and elderly women, provided direct evidence that the quality of bone material differs across age (21). Namely, aged bone matrix showed less elastic behavior in the elderly, adding new experimental insights into the determinants of age-related hip fractures (21).

Taken together, nano-structural evaluation of the bone matrix revealed particular mechanical consequences of the matrix aging, independent from age-related effects at other levels of bone hierarchical structure, that provide new insights into the problem of bone fragility.

References