“GOING NANO” in the field of osteology: Is bone fragility already determined at the level of bone mineralized matrix?

“NANO” u osteologiji: Da li je lomljivost kosti već određena na nivou mineralizovanog koštanog matriksa?

Petar Milovanović, Marija Đurić Srejić

Institut za anatomiju, Medicinski fakultet Univerziteta u Beogradu

Kontakt: drpmilovanovic@gmail.com

Sažetak

Prelomi kostiju su česti kod starijih osoba čak i nakon dejstva sile niskog intenziteta. Budući da se kosti mlade osobe u takvim uslovima ne bi slomile, jasno je da glavni uzrok povećane koštane lomljivosti potiče od strukturnih i fizičko-hemijskih karakteristika same stare kosti. Ispitivanje kostiju od makro- do nano-nivoa oslikava svu složenost koštane hijerarhijske organizacije i ukazuje na različite faktore od kojih zavisi otpornost kosti na prelome. Posebno su značajne skorašnje studije koje su se usredsredile na ispitivanje kosti na nanometarskom nivou i otkrile konkretne parametre koštanog matriksa čije poznavanje doprinosi boljem razumevanju povećane koštane fragilnosti kod starijih osoba, nezavisno od starosnih promena na drugim nivoima koštane organizacije.

Ključne reči: Kost, fraktura, nano, starenje, mineralni kristali

Abstract

Bone fractures frequently occur in elderly persons even after low-energy trauma. Given that the young individuals would not sustain a fracture under such conditions, it is clear that the main cause of easy bone fracturing originates from the structural and compositional characteristics of the aged bone itself. Observing bone, from macro- to nano-level, shows us the complexity of bone hierarchical organization and reveals various determinants of bone strength. In particular, recent studies focusing on bone at nano-scale revealed distinctive features of the bone matrix that could provide additional explanation for the increased bone fragility in advanced age, independent from age-related effects at other levels of bone hierarchical structure.

Key words: Bone, nano, fracture, aging, mineral crystals

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 anchor-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 predi-

lection 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-

Figure 1. Bone structural organization: Macroscopic (A-B), microscopic (C-D), and nano-level (E-F). (A) A view on the proximal half of a human femur (white line shows the location of the section in B); (B) Section of the femoral neck showing cortical (outer) and trabecular (inner) bone compartments. (C) Microscopic view (SEM) of trabecular bone showing multiple interconnected trabeculae. (D) Microscopic view (backscatter SEM) of cortical bone showing numerous osteons (white circle) with Haversian canals (white asterisk) in the middle. (E) Longitudinal view (schematic) on collagen fibrils covered by mineral crystals. (F) Transversal section of collagen fibrils (schematic). Note that mineral crystals lie both between and inside the collagen fibrils (interfibrillar and intrafibrillar mineral). Non-collagenous proteins are not shown here for simplicity.

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 net-

work 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

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

Abbreviations: Raman – Raman spectroscopy, FTIR – Fourier transform infrared spectroscopy, EDS – Energy dispersive X-ray spectroscopy, SEM – Scanning electron microscopy, AFM – Atomic force microscopy, AFM-PSD – AFM-based power spectral density, XRD – X-ray diffraction.

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, understand-

ing 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.

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

1. Van Staa TP, Dennison EM, Leufkens HGM, Cooper C. Epidemiology of fractures in England and Wales. *Bone* 2001;29(6):517-22.
2. Cooper C, Campion G, Melton LJ. Hip fractures in the elderly: A world-wide projection. *Osteoporos Int* 1992;2(6):285-9.
3. Banks E, Reeves GK, Beral V, Balkwill A, Liu B, Roddam A, et al. Hip Fracture Incidence in Relation to Age, Menopausal Status, and Age at Menopause: Prospective Analysis. *PLoS Med* 2009;6(11):e1000181.
4. Cole Z, Dennison E, Cooper C. Osteoporosis epidemiology update. *Curr Rheum Rep* 2008;10(2):92-6.
5. Currey JD. *Bones: structure and mechanics*. Princeton, N. J.: Princeton University Press; 2002.
6. Bonucci E. Basic composition and structure of bone. In: An YH, Draughn RA, editors. *Mechanical testing of bone and the bone-implant interface*. Boca Raton: CRC Press; 2000. p.3-22.
7. Jee WSS. Integrated bone tissue physiology: Anatomy and Physiology. In: Cowin SC, editor. *Bone mechanics handbook*. Boca Raton: CRC Press; 2001. p.1-68.
8. Jobke B, Milovanovic P, Amling M, Busse B. Bisphosphonate-osteoclasts: Changes in osteoclast morphology and function induced by antiresorptive nitrogen-containing bisphosphonate treatment in osteoporosis patients. *Bone* 2014;59:37-43.
9. Milovanovic P, Zimmermann EA, Hahn M, Djonic D, Püschel K, Djuric M, et al. Osteocytic Canalicular Networks: Morphological Implications for Altered Mechanosensitivity. *ACS Nano* 2013;7(9):7542-51.
10. Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J. Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone* 2007;41(5):745-51.
11. Turner PJ. Atomic force microscopy and indentation force measurement of bone. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2009;1(6):624-49.
12. Boskey AL. Bone mineralization. In: Cowin SC, editor. *Bone mechanics handbook*. Boca Raton: CRC Press; 2001. p5/1-5/33.
13. Xin R, Leng Y, Wang N. HRTEM Study of the mineral phases in human cortical bone. *Adv Eng Mater*. 2010;12(9):B552-B7.
14. Fantner G, Hassenkam T, Kindt JH, Weaver JC, Birkedal H, Pechenik L, et al. Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture. *Nat Mater* 2005;4:612.
15. Currey J. The structure and mechanics of bone. *J Mater Sci* 2012;47(1):41-54.
16. Currey JD. The many adaptations of bone. *J Biomech* 2003;36(10):1487-95.
17. Roschger P, Paschalis EP, Fratzl P, Klaushofer K. Bone mineralization density distribution in health and disease. *Bone* 2008;42(3):456-66.
18. McCreddie BR, Morris MD, Chen T-c, Sudhaker Rao D, Finney WF, Widjaja E, et al. Bone tissue compositional

- differences in women with and without osteoporotic fracture. *Bone* 2006;39(6):1190-5.
19. Milovanovic P, Zimmermann EA, Riedel C, Scheidt Av, Herzog L, Krause M, et al. Multi-level characterization of human femoral cortices and their underlying osteocyte network reveal trends in quality of young, aged, osteoporotic and antiresorptive-treated bone. *Biomaterials* 2015;45(0):46-55.
 20. Hassenkam T, Fantner GE, Cutroni JA, Weaver JC, Morse DE, Hansma PK. High-resolution AFM imaging of intact and fractured trabecular bone. *Bone* 2004;35(1):4-10.
 21. Milovanovic P, Potocnik J, Djonic D, Nikolic S, Zivkovic V, Djuric M, et al. Age-related deterioration in trabecular bone mechanical properties at material level: Nanoindentation study of the femoral neck in women by using AFM. *Exp Gerontol* 2012;47(2):154-9.
 22. Kindt JH, Fantner GE, Thurner PJ, Schitter G, Hansma PK. A new technique for imaging mineralized fibrils on bovine trabecular bone fracture surfaces by atomic force microscopy. *Mater Res Soc Symp Proc* 2005;874:59-65.
 23. Fantner GE, Rabinovich O, Schitter G, Thurner P, Kindt JH, Finch MM, et al. Hierarchical interconnections in the nano-composite material bone: Fibrillar cross-links resist fracture on several length scales. *Compos Sci Technol* 2006;66(9):1202-8.
 24. Kindt JH, Thurner PJ, Lauer ME, Bosma BL, Schitter G, Fantner GE, et al. In situ observation of fluoride-ion-induced hydroxyapatite-collagen detachment on bone fracture surfaces by atomic force microscopy. *Nanotechnology* 2007;18(13):135102.
 25. Thurner PJ, Oroudjev E, Jungmann R, Kreutz C, Kindt JH, Schitter G, et al. Imaging of bone ultrastructure using atomic force microscopy. In: Méndez-Vilas A, Díaz J, editors. *Modern research and educational topics in microscopy*. Badajoz: Formatex; 2007. p.37-48.
 26. Sasaki N, Tagami A, Goto T, Taniguchi M, Nakata M, Hikiuchi K. Atomic force microscopic studies on the structure of bovine femoral cortical bone at the collagen fibril-mineral level. *J Mater Sci Mater Med* 2002;13(3):333-7.
 27. Milovanovic P, Rakocevic Z, Djonic D, Zivkovic V, Hahn M, Nikolic S, et al. Nano-structural, compositional and micro-architectural signs of cortical bone fragility at the superolateral femoral neck in elderly hip fracture patients vs. healthy aged controls. *Exp Gerontol* 2014;55:19-28.
 28. Milovanovic P, Djuric M, Neskovic O, Djonic D, Potocnik J, Nikolic S, et al. Atomic force microscopy characterization of the external cortical bone surface in young and elderly women: potential nanostructural traces of periosteal bone apposition during aging. *Microscopy and Microanalysis* 2013;19(5):1341-9.
 29. Milovanovic P, Djuric M, Rakocevic Z. Age-dependence of power spectral density and fractal dimension of bone mineralized matrix in AFM topography images: Potential correlates of bone tissue age and bone fragility in female femoral neck trabeculae. *J Anat* 2012;221(5):427-33.
 30. Milovanovic P, Potocnik J, Stoiljkovic M, Djonic D, Nikolic S, Neskovic O, et al. Nanostructure and mineral composition of trabecular bone in the lateral femoral neck: Implications for bone fragility in elderly women. *Acta Biomater* 2011;7(9):3446-51.
 31. 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.