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The Role of Matrix Composition in the Mechanical Behavior of Bone

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Abstract

Purpose of Review While thinning of the cortices or trabeculae weakens bone, age-related changes in matrix composition also lower fracture resistance. This review summarizes how the organic matrix, mineral phase, and water compartments influence the mechanical behavior of bone, thereby identifying characteristics important to fracture risk.

Recent Findings In the synthesis of the organic matrix, tropocollagen experiences various post-translational modifications that facilitate a highly organized fibril of collagen I with a preferred orientation giving bone extensibility and several toughening mechanisms. Being a ceramic, mineral is brittle but increases the strength of bone as its content within the organic matrix increases. With time, hydroxyapatite-like crystals experience carbonate substitutions, the consequence of which remains to be understood. Water participates in hydrogen bonding with organic matrix and in electrostatic attractions with mineral phase, thereby providing stability to collagen-mineral interface and ductility to bone.

Summary Clinical tools sensitive to age- and disease-related changes in matrix composition that the affect mechanical behavior of bone could potentially improve fracture risk assessment.

Keywords Mineral · Type 1 collagen · Bone quality · Advanced glycation end-product · Post-translation modifications · Water

Introduction

The factors contributing to the age- and disease-related increase in fracture risk are multifactorial and include deleterious changes to the compositional characteristics of the bone matrix. Since there are currently no clinical tools for directly assessing these characteristics in patients, much of the knowledge regarding the role of matrix composition in the fracture resistance of bone comes from studies of cadaveric bone, discarded bone acquired at the time of surgery, and iliac crest biopsies as well as from rodent studies of aging and diseases that affect the bone (e.g., diabetes, osteogenesis imperfecta, loss of matrix-associated genes). In such studies, matrix properties were either correlated with the age-related decrease in material properties of bone as determined by a variety of mechanical tests [1] or reported as differences in tissue-level compositional and mechanical properties between a control group and a osteoporotic group [2].

The measurement of areal bone mineral density (aBMD) from dual-energy X-ray absorptiometry (DXA) remains the gold-standard for deciding whether an individual has osteoporosis (ie, a T-score $\leq$ –2.5). While the use of clinical risk factors (FRAX) and trabecular bone score (i.e., texture analysis of DXA images of the lumbar spine) improve the prediction of fracture risk over T-scores alone [3], fragility fractures still occur in seemingly low risk individuals [4]. As recently described in a review article [5], several advances in imaging technologies have been developed to overcome the limitations of the projected measurement of aBMD and potentially improve fracture risk assessment beyond statistical models based on epidemiological studies. To date, these technologies − from finite element analysis derived from quantitative computed

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tomography (QCT) images of the hip or spine to assessments of cortical structure and trabecular architecture at peripheral sites − have indicated that thinning of the cortices [6], increases in cortical porosity [7], and deterioration in trabecular architecture [8] all likely contribute to higher fracture risk.

Clinical studies involving the OsteoProbe, a hand-held micro-indentation device, suggest that deleterious changes in the bone matrix also contribute to higher fracture risk. The device provides a measurement of the resistance of a patient's tibia mid-shaft (periosteal surface on the anterior-medial side) to impact loading at a length-scale of ~350 μm. Called Bone Material Strength index (BMSi), this measurement is the depth of a spheroconical tip into bone divided by the depth of the same tip into a standard reference material. As discussed in a recent guidelines paper [9] and a review paper on the technique [10], the compositional factors influencing BMSi are unknown. Nonetheless, several case-control studies have reported lower BMSi for patients with fragility fracture(s) compared with age-matched individuals without a history of low-energy fractures: combined vertebral (n = 8), hip (n = 10), and non-vertebral/non-hip fractures (n = 45) vs non-fracture cases (n = 27) [11], combined vertebral (n = 24), hip (n = 25), and non-vertebral/non-hip fractures (n = 17) vs non-fracture cases (n = 66) [12], and distal radius fractures (n = 57) vs control cases (n = 93) [13•]. However, BMSi was not associated with a history of fracture in one study (fragility not distinguished from high-energy fracture, n = 117 vs non-fracture, n = 63) [14]. Also, the lower BMSi for hip fracture cases (n = 41) compared with control cases (n = 93) did not reach statistical significance ($P = 0.09$) [13•]. Three independent groups reported that post-menopausal women with type 2 diabetes (T2D) had lower BMSi than age-matched women without the disease [15–17]. Since T2D is associated with higher fracture risk for a given T-score [18], the lower BMSi suggests diabetes affects matrix composition, though underlying cortical porosity could be contributing factor. While much remains to be learned about how compositional characteristics affect BMSi and additional studies need to establish the range of BMSi values for healthy bone below which bone is at imminent risk of fracture, the quality of the bone matrix (or lack thereof) likely contributes to increases in fracture risk that occur with aging and certain diseases.

Key attributes of the mechanical behavior of bone include: (1) being stronger in compression than in tension, while experiencing greater post-yield deformation in tension than in compression [19]; (2) having higher resistance to crack growth when a crack propagates perpendicular to the primary direction of osteons (transverse) than when it propagates parallel to osteonal direction (longitudinal) [20]; (3) becoming stiffer but more brittle (lack of toughness) as the strain rate increases over orders of magnitude [21]; (4) exhibiting both microdamage accumulation and creep during fatigue loading (cyclic loading below the yield strength at low frequency over an extended period of time) [22]; and (5) exhibiting viscous dampening at low stress during dynamic loading (cyclic loading at variable frequency over a short period of time) [23]. All these attributes arise not only from the composition of the bone matrix (collagen, mineral, and water) but also from the arrangement of the primary constituents such as the shifting orientation of collagen fibrils, the varying degrees of mineralization (heterogeneity), and the stabilizing interactions between water, hydrophilic residues of peptides, surfaces of mineral crystals, and possibly non-collagenous proteins (NCPs). Bone has numerous mechanisms at multiple length scales to resist the propagation of a crack towards fracture and promote fracture resistance (Fig. 1) such as sacrificial bonds between neighboring mineral crystals (dilatational bands), uncoiling of collagen I, fibril sliding, diffuse and microdamage accumulation, crack deflection at cement lines, fibril bridging of a crack, and uncracked ligament bridging (tortuosity) [24]. The composition of the bone matrix contributes to each of these mechanisms, making bone a remarkable material but also susceptible to multiple deleterious changes that increase fracture risk.

Herein, we describe the role of each of the 3 primary compositional components (mineral, organic matrix, and water) in the mechanical behavior of bone. Of course, the three constituents are interdependent such that the formation of collagen I into fibrils affects mineralization, which in turn can affect the hydration status of the organic matrix.

Proper Collagen Formation and Organization are Required for Post-Yield Toughness

The organic phase of bone is primarily a network of interlinked type I collagen. Specifically, tropocollagen (300 nm × 1.6 nm in diameter) is a triple helix consisting of two α-1 chains and one α-2 chain with a distinct motif (glycine-X-Y)$_n$ in which X is often proline (~28% in collagen I) and Y is often hydroxyproline (~38% in collagen I). Post-translational modifications (PTMs) of collagen are important to the overall structure and stability and, in turn, the mechanical behavior of bone. Hydroxylation of proline is one type of PTM that forms hydroxyproline, which facilitates hydrogen bonding with both water and other amino acids within the collagen chain. Other PTMs such as glycosylation and hydroxylation of lysine facilitate crosslink formation between neighboring collagen molecules [25] with specific enzymes (e.g., lysyl oxidase or lysyl hydroxylase) producing immature enzymatic crosslinks between lysyl or hydroxylysyl residues, which can later be converted to mature enzymatic crosslinks. With this enzymatic crosslinking, the self-aligned collagen molecules are further stabilized. Collagen organization is reviewed in-depth elsewhere [26], but in general, the staggered arrangement of tropocollagen into fibrils generates a periodicity known as a D-band. In atomistic simulations of

Figure 1. Toughening mechanisms in bone exist at multiple hierarchical levels of organization. **A,** PTMs affecting hydroxyl groups may alter the secondary structure of collagen I. **B,** Post-translational modifications to matrix-bound glycoproteins (right) and excessive non-enzymatic crosslinking (left) may favor formation of damage and collagen rupture at the nano-structural level. **C,** Overall changes in collagen structure and hydration at the nanometer scale reduce the ability of bone to prevent cracking, thereby lowering fracture toughness at the material-level. **D,** An increase in porosity (pore water) can also lower fracture resistance
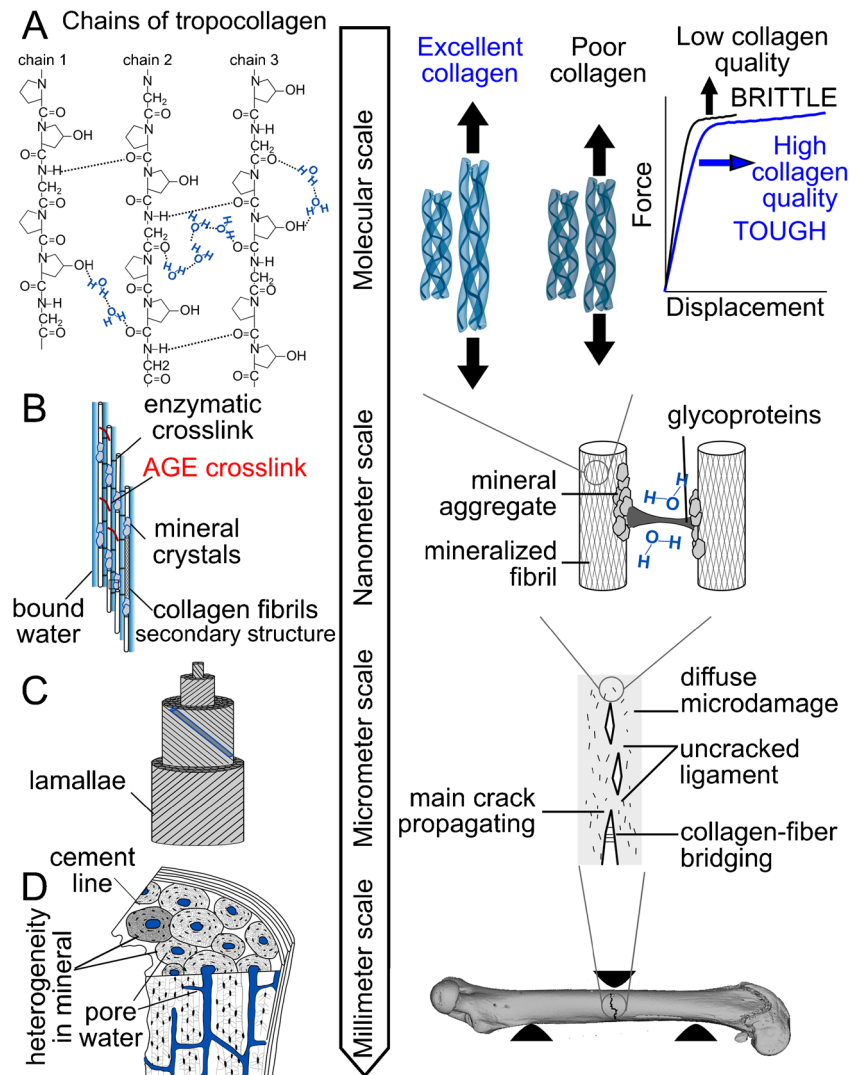
hydrated microfibrils with a D spacing of 67 nm (overlap and gap regions of 0.46D and 0.54D, respectively), the Young's modulus of collagen was determined to be ~300 MPa at small deformation and ~1.2 GP at large deformation (>10% strain) [27]. When mineral is introduced into the model, the tensile modulus increases as a function of increasing mineral content [28•]. D spacing varies among fibrils, and there is experimental evidence that the increase in the toughness of bone upon ex vivo incubation in raloxifene, a small molecule drug, accompanied a shift towards higher values of D-periodic spacing and an increase in matrix hydration [29].

The collagen-rich matrix of bone confers toughness to an otherwise brittle mineral phase (i.e., ceramics like hydroxyapatite exhibit little post-yield deformation). As an example of the importance of collagen to bone toughness, irradiating bovine cortical bone at a high enough gamma dose (33 kGy) to damage the organic matrix (radiolysis) decreased work-to-fracture, post-yield strain, and fracture toughness (n = 8 each group) [30], mechanical properties related to the ductility of

bone. Multiple mouse models of osteogenesis imperfecta (OI) have demonstrated a brittle bone phenotype. OI is a genetic disease in which a variety of mutations can either affect the α-1 chain or α-2 chain of collagen I (dominant) or affect the function of enzymes and chaperones important to collagen I processing and assembly (recessive) [31]. In one example, the deletion of an enzyme, cyclophilin B, a collagen peptidyl-prolyl *cis-trans* isomerase – that is part of a collagen 3-hydroxylation complex – resulted in reduced 3-hydroxylation and lower post-yield displacement, plastic energy, and elastic energy-to-fracture as measured in 4-point bending tests of mouse femurs [32]. Cortical area, ultimate load, and aBMD were lower as well, indicating this model has multiple skeletal defects. Furthermore, accompanying altered fibril structure, there was an overall change in the profile of immature and mature crosslinks with the loss of cyclophilon B [32]. Proper formation of the tropocollagen and then proper PTMs and subsequent crosslinking are required for bone to deform after yielding or the onset of damage.

Collagen Fibril Orientation Influences Strength and Fracture Toughness

Moving up the hierarchical arrangement of the organic matrix to the micron-scale, the orientation of collagen fibrils changes from one lamellae to the next (Fig. 1). This altering orientation gives rise to oscillating modulus values across lamellae as observed by scanning acoustic microscopy measurements of cortical bone sections [33•, 34]. Raman spectroscopy (RS) mapping of the lamellae (Amide I) indicated that the oscillating elasticity at the tissue-level is due to alternating fibril orientation as opposed to alternating mineralization [33•]. The varying fibril orientations across lamellae increases the energy that must be expended to propagate a crack [35]. In a study combining mechanical tests of cortical micro-beams (focus ion beam followed by loading with the tip of an atomic force microscope) and computational mechanics, fibril orientation correlated with calculated bone strength (n = 6) [36]. As an example of the importance of fibril organization to bone toughness, the brittle bone phenotype of mice lacking activating transcription factor 4 (ATF4) was not necessarily due to differences in tissue mineral density but rather to an apparent difference in fibril orientation between ATF4 knock-out (n = 14) and wild-type littermates (n ≥ 14) [37]. Specifically, the change in the prominent phosphate peak (RS) per Amide I peak upon rotating the femur mid-shaft 90 degrees relative to the polarization axis of the laser was dissimilar between the genotypes. To clarify, collagen molecules and mineral crystals in the bone matrix are birefringent, and as such, can change the primary orientation of the incident light. Since Raman microscopes are sensitive to polarization, even without added optics to explicitly polarize the laser (i.e., to cause virtually one orientation of the light), the height of the Amide I peak depends on preferred collagen fibril orientation. As another example, in a transgenic mouse model of steroid-induced osteoporosis (n = 5), there was an increase in the randomness of fibril orientation and an increase in fibril strain for a given tissue strain as well as reduced mineralization, compared with the wild-type mice (n = 5), culminating in lower tensile breaking stress of anterior cortices (femur) [38]. The effect of fibril orientation on mechanical properties is not necessarily independent of mineralization as the long axis of HA crystals align with the long axis of collagen fibrils.

Mature Enzymatic Collagen Crosslinks Stabilize the Matrix Providing Strength to Bone

Collagen crosslinks add stability to the organic matrix, preventing micro-fibrils from sliding past one another. In a mouse model of lathyrism (lysyl oxidase inhibition by a toxin), there was a positive correlation (1) between ability of cortical bone to resist crack growth (notch created in the femur diaphysis to assess fracture toughness) and the ratio of mature to immature enzymatic crosslinks (indirectly by RS by Amide I sub-peak ratio, 1660/1683,) ($R^2 = 0.208$, $P < 0.05$, n ≥ 28), and (2) between bending strength (tibia diaphysis) and mature pyridinoline crosslinks ($R^2 = 0.159$, $P < 0.05$, n ≥ 28) [39••]. Furthermore, the lower bending strength and lower fracture toughness for the mice administered the toxin compared with the controls occurred without a difference in tissue mineral density. In a study comparing adolescent bone (n = 7) to elderly bone (n = 3), the ratio of mature to immature enzymatic crosslinks (directly by high performance liquid chromatography or HPLC) increased in humans, but the post-yield toughness decreased (distal fibula) [40]. While there were differences in the mechanical testing between the mouse and human study (bending of whole mouse bone compared with bending of machined cortical samples), the discrepancy is partially due to the difference in experimental design. Disrupting enzymatic crosslinking as in McNerny et al. likely lead to changes in collagen structure, thereby decreasing the fracture resistance of bone (n ≥ 7 per group) [39••], but in normal physiology, these enzymatic crosslinks mature, increasing the mature to immature ratio, with skeletal maturation. Along with the increase in mineralization that also occurs with skeletal maturation, adult bone with higher mature collagen crosslinks, including those formed non-enzymatically, loses the initial high post-yield deformation capacity of adolescent bone. In an aging rat study (n = 12 per age group) with little remodeling, the loss in bone toughness increases in mature crosslink concentrations (HPLC) but also increases in tissue mineral density [41].

Non-Enzymatic Collagen Crosslinking Can Lower Bone Toughness

While enzymatic crosslinks may confer stability to the organic matrix, increases in non-enzymatic crosslinks are thought to have an embrittling effect on the matrix. Non-enzymatic collagen crosslinks, a type of advanced glycation end-product (AGE), are usually quantified by either HPLC to measure pentosidine concentration or a fluorescence assay to measure total fluorescent AGEs (fAGEs), though a recent study identified a FTIR measure of non-enzymatic crosslinking in bone [42]. Pentosidine can be viewed as a marker for total fAGEs, but there are limitations to when it correlates with fAGEs, which is nonspecific measurement [43]. AGEs accumulate in the bone matrix with age, and as AGEs increase, the toughness of cortical bone decreases [1]. In a study that incubated bovine cortical bone in a high concentration of ribose, pentosidine increased while post-yield strain and flexural toughness decreased (n = 15 per group, non-incubated control, incubated control, and glycated groups) [44]. Interestingly, differential scanning calorimetry determined that the glycated samples

had a higher thermal stability as indicated by a higher thermal denaturation onset temperature (T_{onset}), and scanning electron microscopy images of the tensile side of the beam specimens showed a smoother fracture surface suggesting reduced toughening mechanisms [44]. Decreases in microcrack density and increases in crack length with glycation have been observed in another ribose study involving human cortical bone (n = 9 per group, control vs glycated) [45•]. When porcine cancellous bone was incubated in ribose, tissue-level stiffness as measured by nanoindentation did not alter despite an increase in pentosidine (n = 12 per group, control vs glycated) [46]. In a transgenic mouse model of early-onset, severe type 1 diabetes, OVE26 mice (n = 6), pentosidine was higher while fracture toughness parameters were lower compared with the non-diabetic FVB mice (n = 6) [47]. There are also other abundant AGEs in bone, including carboxymethyllysine, which is an adduct, not a crosslink. Overall, there is a paucity of information on whether such AGEs plus non-fluorescent AGEs (e.g., glucosepane) affect the mechanical behavior of bone. Moreover, there is no in vivo evidence, to date, that blocking AGE accumulation rescues the loss in bone toughness with aging and the onset of diabetes.

Non-Collagenous Proteins Contribute a Toughening Mechanism at the Nano-Scale

While collagen I is the most abundant protein in the organic matrix (~90%), non-collagenous proteins (NCPs) likely also contribute to fracture resistance of bone. NCPs such as osteopontin influence mineralization, and when carboxylated, they become trapped in the matrix. These proteins create sacrificial bonds that can dissipate energy and provide a toughening mechanism to bone at the nano-scale level of organization [48]. Using atomic force microscopy and confocal laser microscopy imaging of bone specimens subjected to fatigue loading, the identification of dilatational bands (i.e., formation of small voids between mineral crystals following fatigue loading) were associated with NCPs [49]. The role of NCPs in the fracture resistance of bone has been primarily based on knock-out mouse models [1]. More recently, in a model of accelerated aging, the α-klotho-/- mouse (n = 4), there was less stiffening in dynamic nanoindentation tests (i.e., less of an increase in storage modulus with an increase in frequency of loading), compared with wild-type mice (n = 4) [50]. The authors suggested that the known reduction osteocalcin in this model caused a loss of dilatational bands to form under dynamic loading. For mice deficient in both osteocalcin and osteopontin (n = 8), fracture toughness (femur diaphysis) was lower compared with control femurs (n = 8) [49], but the structural strength (radius diaphysis) was higher because of a higher cortical area that occurred in the double knock-outs [51]. Thus, global deletion of NCPs can have multiple effects

beyond tissue composition, and so unravelling their role in fracture resistance is challenging. More studies involving human bone are needed to determine their contribution to bone fragility relative to other established toughening mechanisms that are affected by age and disease.

Subjected to Imperfections to Its Structure, Bone Mineral Confers Strength

The mineral phase of bone is mainly composed of calcium phosphate in the form of nano-sized crystals of hydroxyapatite (HA). Differing from synthetic HA, bone mineral incorporates carbonate (5%–8%) over time substituting for phosphate (type-B) and for hydroxyl groups (type-A) within the crystal lattice [52]. With other trace cations (e.g., Mg^{2+}, K^{1+}, Na^{1+}) and trace anions (e.g., F^{1-}, Cl^{1-}) substituting for calcium, phosphate, or hydroxyl groups over time as well as citrate and water filling vacancies in the lattice [53], the crystal structure of bone mineral is an imperfect plate (5 nm × 70 nm × >200 nm) rather than the prismatic shape of a HA crystal [54]. Thus, bone mineral is carbonated HA with varying crystallinity and varying substitutions within the lattice.

Mineral primarily contributes to the strength and elastic modulus of bone such that these mechanical properties increase as ash fraction [55] or degree of mineralization [56] increase. Using wide-angle X-ray scattering/small-angle X-ray scattering (WAXS/SAXS) techniques coupled with in situ compressive loading of bone, recent studies further established that mineral mainly carries compressive load in bone [57, 58]. In addition to mineral quantity, its distribution spatially and its quality (i.e., mineral maturity/crystallinity and carbonate substitutions) likely influences the mechanical behavior of bone, though less is known about the role of mineral quality in the mechanical behavior of bone.

Increases in Mineralization Can Promote Strength but Too Much Mineralization Hinders Fracture Resistance

Various techniques exist to quantify degree of mineralization, including quantitative microradiography (qMR), quantitative backscattered electron imaging (qBEI), micro-computed tomography (μCT), Fourier transform infrared spectroscopy (FTIR), and RS [59]. Unlike qBEI-derived mineralization (Ca-Peak) or μCT-tissue mineral density (mgHA/cm^3), mineral-to-matrix ratio (MMR) from FTIR or RS provides the amount of mineral per amount of organic matrix [60]. Several rodent studies have reported direct correlations between MMR and bone strength (aging) (n = 13) [61] and between MMR and tissue tissue-level modulus (vitamin D deficiency) (n = 10) [62]. In rodents, Ca content and tissue-

level modulus and hardness concomitantly increase through skeletal maturity (6–7 months) but do not vary much with aging (between 7 months and 17 months, n = 7/age group) [63]. Tissue mineral density has been observed to increase in male rats between 12 months and 24 months without any change in bending strength (n = 12/age group) [41], suggesting other factors also influence strength of bone. Thus, increasing mineralization of maturing bone confers an increase in elastic properties, including modulus at the tissue level and strength at the apparent level.

A decrease in the degree of mineralization with osteoporosis is typically the result of elevated bone turnover [64], and associations between various measurements of mineralization, and fracture risk can be found in a previous review [2]. In a recently published study involving embedded and sectioned iliac crest biopsies from age- and aBMD-matched post-menopausal subjects with (n = 60) and without history of fracture (n = 60), Vennin et al. [65] found that the median value of tissue-level modulus and hardness (nanoindentation) of only the cortical bone was lower for the fracture than for the non-fracture cases. Interestingly, there were no significant correlations between the nanoindentation properties and MMR (FTIR). In another recent biopsy study, patients on long-term bisphosphonates (BPs) who experienced an atypical subtrochanteric fracture (n = 17) had higher MMR (FTIR and RS but not Ca-Peak by qBEI-derived mineralization) and higher tissue-level hardness (nanoindentation) than patients on long-term BPs with typical femoral neck fractures (n = 10) or BP-naïve patents with typical fracture (n = 11) or without fracture (n = 12) [66••]. In mature human bone undergoing normal remodeling, the contribution of local mineralization to apparent-level modulus and strength is less important as microstructure (e.g., cortical porosity) dictates strength, but in certain cases (e.g., suppressed remodeling), loss of heterogeneity can become a dominant factor in which high mineralization relative to the organic matrix reduces fracture resistance.

Based on genetic mouse and iliac biopsy studies, degree of mineralization (qBEI) is known to increase in OI [67], likely due to increased packing density of crystals, not an increase in crystal size [68]. As reported in a recent study involving pediatric cortical bone from mild to severe OI, tissue mineral density (μCT) and MMR (RS) were higher, while tissue-level modulus (nanoindentation) was lower in OI individuals (n = 7) compared with control group (n = 3) [69], indicating a disruption in the structure–function relationship between elastic behavior and mineralization. In a traditional genetic mouse model of OI (OIm mice, n = 20, that produce only the $\alpha 1$ chain), the higher degree of mineralization, compared with control bone (n = 15), also accompanied a reduced tissue-level modulus (nanoindentation) [70] as well as a decrease in fracture toughness (n = 10 per group) [71••]. In the mouse model of OI involving deletion of prolyl 3-hydroxylase 1, important for PTMs to proline, there was an increase in bone mineralization

(qBEI) (n = 16 per group) [72] but also abnormalities in the collagen fibril ultrastructure (n = 10 per group) [73]. Thus, the apparent hypermineralization in OI likely contributes to brittle bone disease but so do defects in the collagen structure.

Mineralization Heterogeneity Contributes to a Toughening Mechanism

Mineralization heterogeneity refers to the spatial variation in the degree of mineralization throughout bone at the micron length scale and is typically assessed as full width at half maximum (FWHM) of the distribution of mineralization levels (e.g., by qBEI, FTIR imaging, or qMR). Studies of human bone samples observed both higher [74, 75] and lower [76, 77] mineralization heterogeneity for fragility fracture cases compared with control cases. Based on computational mechanics [78], the presence of mineralization heterogeneity at the microscale level increases the energy to propagate a crack, thereby providing bone's ability to resist crack propagation (cement line density also increased fracture toughness). However, increasing mineralization heterogeneity arises from an increase in remodeling and the accompanying increase in porosity can lower fracture toughness [79•]. A recent fatigue study of trabecular bone involving sequential labeling of damage also indicated that compositional heterogeneity favored propagation of microdamage within center of trabeculae, not at regions of high stress (surface of trabeculae) [80•]. Therefore, there is likely both an optimal heterogeneity in mineralization (as well as in fibril morphology) and an optimal spatial distribution of varying mineralization (as well as in varying fibril orientation) that effectively promotes fracture resistance.

How Crystallinity/Mineral Maturity Contributes to Mechanical Behavior of Bone is not Known

Crystallinity is an overall indicator of crystal size and crystal lattice perfection (i.e., the degree of order of the ions within the crystal lattice) of mineral [81]. Mineral maturity, on the other hand, refers to the transformation of unstable non-apatitic substance into more crystalized stable mineral, reflecting the age of bone mineral [82]. X-ray diffraction techniques provide direct information on the size, orientation, and chemical composition of bone crystals, while FTIR (sub-band area ratio at 1030 cm^{-1} and 1020 cm^{-1}) and RS (full width at half maximum of the $v_1 PO_4$ band) provide indirect measures of mineral maturity/crystallinity. In a genetic mouse model of matrix metalloproteinase deletion (MMP-2), Bi et al. [83] reported that crystallinity (RS) was directly proportional to bending modulus ($R^2 = 0.64$, $P < 0.05$, n = 36) and strength

($R^2 = 0.40$, $P < 0.05$, n = 36). In a study involving human cadaveric cortical bone specimens, Yerramshetty and Akkus [84] found that crystallinity (RS) was directly proportional to elastic modulus ($R^2 = 0.16$, $P = 0.001$, n = 64) and yield strength ($R^2 = 0.07$, $P = 0.039$, n = 64) when all data were pooled. Of course, these associations do not demonstrate that an increase in crystallinity directly increases material strength of bone, as other factors of the bone matrix can influence the mechanical behavior. For human bone (117 bone biopsies from 40 females and 77 males between 0 and 90 years old), Hanschin and Stern found that crystallinity (X-ray diffraction) was observed to increase up to 25 years of age while it did not vary in individuals between 30 and 80 years old [85], an age range in which fracture resistance declines. Thus, crystallinity is likely not a major contributor to the age-related decrease in the mechanical properties of bone, but it could be a biomarker of diseased bone.

For example, crystallinity (RS) was found to be lower with a corresponding reduction in indentation modulus for bone samples from OI subjects (n = 7) than from age-matched controls (n = 3) [69]. In an iliac bone biopsy study involving long-term treatment of postmenopausal osteoporosis with alendronate (6–10 years) and matching for the degree of mineralization across the groups, the treated group (n = 6) had significantly lower crystallinity compared with the BP-naïve group (n = 5) [86]. Moreover, crystallinity (FTIR) was negatively associated with tissue-level modulus and hardness in only the treated group ($R^2 = 0.18$ and $R^2 = 0.29$, $P < 0.001$, respectively, n = 6) when including the amount of mineral and collagen maturity (FTIR) as covariates. There is no data to date showing whether there is an optimal crystal size associated with adequate bone strength while maintaining bone ductility. However, one hypothesis is that heterogeneity in crystal perfection (i.e., wide distribution in crystal sizes and substitutions) favors adequate bone strength [87].

Carbonate Substitution Increases with Aging and May Negatively Affect Fracture Resistance

Carbonate substitution within the bone mineral lattice is thought to create internal strains in the matrix and increase the irregularity of the atomic arrangement of HA (i.e., Ca^{2+}, PO_4^{3-}, and OH^-) [82]. Thus, such substitutions may limit crystal growth by increasing the required energy for the process [88], consequently altering the length and thickness of the bone mineral crystal. Type B carbonate substitutions (CO_3/PO_4 by RS) have been found to correlate with bone mechanical properties. For example, this ratio inversely correlated with bending modulus and yield strength ($R^2 = 0.33$ and $R^2 = 0.23$, $P < 0.05$, n = 13) of rat femurs tested in three-point bending [61] and inversely correlated with crack growth toughness of human cortical bone acquired from donors (n =

62) spanning 21–101 years of age [89]. Again, establishing the causal role of carbonate substitutions in the mechanical behavior of bone is rather difficult. One possibility is that carbonate substitution may control bone crystal size such that high carbonate concentration results in smaller crystals [88]. Increasing substitutions then would limit the number of interactions between mineral and collagen I, thereby increasing tissue-level modulus (mineral is stiffer than collagen) while decreasing toughness (less energy dissipated from mineral-collagen separation).

Matrix-Bound Water Promotes While Pore Water Hinders Mechanical Properties of Bone

Water is an abundant component of bone and exists in three different compartments [90]: (1) structural solid-like water as a part of the mineral lattice or integrated into the tropocollagen ultrastructure; (2) pore water (also referred to as mobile, unbound, or free water) within the Haversian canals, canaliculi, and lacunae, and (3) bound water arising from hydrogen bonding (collagen) and electrostatic attractions (mineral) with various degrees of affinity, ranging from loosely to tightly bound states. Dehydration of bone causes an increase in stiffness at multiple length scales but an overall decrease in toughness [90]. In effect, matrix-bound water provides ductility to the organic matrix allowing the collagen to extend beyond the yield point of bone. When water is removed, collagen contracts, increasing the apparent stiffness of bone.

With the application of ^1H nuclear magnetic resonance (NMR) relaxometry [91] and its translation to clinical magnetic resonance imaging (MRI) with ultra-short time-to-echo techniques [92], the independent role of different water compartments in the mechanical behavior of hydrated bone is now beginning to be investigated. Both pore water (negative) and bound water (positive) independently explain the variance in mechanical properties of bone [91–94]. Unal et al. recently implemented high wave number RS system to assess bound water in bone at the molecular level with the ability of probing both collagen- and mineral-bound water simultaneously [95]. In a follow-up study using this new technique, collagen-bound water measurement from hydrated bovine cortical bone significantly correlated with toughness ($R^2 = 0.52$, $P = 0.001$, n = 30), post-yield toughness ($R^2 = 0.44$, $P < 0.001$, n = 30), and bending strength ($R^2 = 0.26$, $P < 0.001$, n = 30) [96].

While pore water is essentially a surrogate measure of cortical porosity because of a strong correlation between these two parameters [91, 97], the important factors affecting bound water with respect to mechanical behavior of bone is less clear. Matrix-bound water is likely important to the post-yield behavior of bone via its inter-relationship with the organic matrix. As shown in a recent study, enzymatic treatment of human cortical bone surfaces to remove glycosaminoglycans (GAGs)

caused a significant decrease in tissue-level toughness (nano-scratch testing) only when water was present [98]. In another recent study, bound water of human cortical bone increased following high dose of radiation exposure and following rotating fatigue testing [99]. A RS-derived Amide I sub-peak ratio also increased following these manipulations, suggesting that gamma radiation-induced matrix damage increased the number of hydrogen bonding sites to which water could interact. It is still unclear how bound water decreases with aging but could involve a loss in matrix-bound glycoproteins and proteoglycans and/or unfavorable modifications in collagen I.

Conclusion

In addition to the known contributions of the organic matrix (type 1 collagen) and the mineral phase (carbonated hydroxyapatite) to the toughness and strength of bone, respectively, the ultrastructure of these constituents and their shifting arrangement throughout bone limit accumulation of damage and propagation of this damage into a fracture (Fig. 1). While disruptions in post-translation modifications that affect fibril organization and mineralization cause a loss in the mechanical properties of bone, identifying the key age- and disease-related changes in matrix composition and organization remain a challenge, especially with respect to the clinical assessment of the matrix contribution to fracture risk. There are, however, emerging candidates for markers of poor bone matrix quality. For example, increase in AGEs, decrease in matrix-bound water, increase in carbonate substitutions, and excessive mineralization have been associated with low toughness and low fracture toughness. The loss of heterogeneity in mineralization and in varying fibril orientation is also indicative of poor fracture resistance. Moving forward, developing a way to measure the consequence of all these deleterious changes, namely alterations in the helical structure of collagen I, arrangement of fibrils, and collagen-mineral-water interactions, could provide a functional assessment of how changes in matrix composition affect the ability of bone to resist fracture.

Compliance with Ethical Standards

Conflict of Interest Jeffry Nyman reports grants for the following: NIH/NIAMS AR063157, NIH/NIAMS AR067871, VA BLR&D BX001018, and NSF 1068988, during the conduct of the study; non-financial support from ActiveLife Scientific, Inc.; and holds a patent, US Patent 8,923,948 System, pertaining to the measurement of bound water and pore water as an indicator of fracture risk (not licensed and no royalties received).

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Mustafa Unal reports no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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