Accepted version of the manuscript published in final form at Nature Reviews Materials (2016) doi:10.1038/natrevmats.2016.41

A materials science vision of extracellular matrix mineralization

N. Reznikov[1,2,3], J.A.M. Steele[4], P. Fratzl[5] and M. M. Stevens[1,2,3,4]

1 Department of Materials, Imperial College London, London, SW6 7PB, UK.

2 Department of Bioengineering, Imperial College London, London, SW6 7PB, UK.

3 Institute of Biomedical Engineering, Imperial College London, London, SW6 7PB, UK.

4 Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, SE 171 77, Sweden.

5 Department of Biomaterials, Max Planck Institute of Colloids and Interfaces, Potsdam-Golm, 14476, Germany.

Corresponding author: M.M. Stevens, m.stevens@imperial.ac.uk

Abstract

From an engineering perspective, skeletal tissues are remarkable structures in that they are lightweight, stiff and tough, yet produced at ambient conditions. The biomechanical success of skeletal tissues is largely attributable to the process of biomineralization – a tightly regulated, cell-driven formation of billions of inorganic nanocrystals formed from ions found abundantly in body fluids. In this Review, we discuss nature's strategies to produce and sustain appropriate biomechanical properties in mineralizing (by promotion of mineralization) and nonmineralizing (by inhibition of mineralization) tissues. We review how perturbations of biomineralization are controlled over a continuum which spans from the desirable (or defective in disease) mineralization of the skeleton, to pathological cardiovascular mineralization, and to mineralization of bioengineered constructs. A materials science vision of mineralization is presented with an emphasis on the micro- and nanostructure of mineralized tissues recently revealed by state-of-the-art analytical methods, and on how biomineralization-inspired designs are impacting the field of synthetic materials.

Web summary

Complex mechanisms are at play in the biomineralization of skeletal tissues and in patholological calcification in the cardiovascular system. In this Review, the physiochemical and biomechanical properties of mineralized tissues, both physiologic and pathophysiologic, and analytical methods to elucidate their finer structure are discussed.

Introduction

Most processes and structures in nature follow the principle of 'minimum inventory – maximum diversity'[1]. The diversity of natural forms is even more astonishing if we consider their multifunctionality as a design strategy[2]. Biomineralization is a fundamental example of how a narrow selection of biologically-available elements is used for metabolic and physiological processes, and also provides elegant engineering solutions to biomechanical challenges. Indeed, calcium and phosphate ions are among the most important ions in our body.

Calcium is used in muscle contraction, nerve signal transmission and in blood clotting,[3] and phosphate serves as a basis for the metabolic pathway between adenosine di- and triphosphate conversions as well as being a potent route by which proteins are modified specifically for cell signalling[4] and for binding to mineral crystals in the body[5, 6]. The concentrations of calcium and phosphate ions are extremely tightly regulated in blood serum, in the extracellular tissue fluid, and intracellularly. Bone is the essential storage depot for these ions in the form of crystallites of calcium-phosphate mineral (a carbonated form of hydroxyapatite), which together with the organic matrix fulfils the mechanical functions of the skeleton by providing support, locomotion and protection. The mechanical tasks of bone require stability and strength combined with the capacity to grow and self-repair, however, the physiological roles of ionic calcium and phosphate require high reactivity and turnover, and fine regulation. Finally, both the biomechanical and metabolic roles rely on efficient transport, fast mobilization and appropriate sequestration of calcium and phosphate ions without inducing the skeleton to dissolve, and without hardening soft tissues. This phenomenon raises two important connected questions, which are not only of medical relevance but of major interest to materials chemistry: how are the aforementioned processes coordinated and regulated appropriately in a living organism, and how does their perturbation link to pathological conditions (Table 1)? This Review addresses these questions from a materials science perspective. We will outline the analytical techniques used to study mineralized and nonmineralized tissues in healthy and diseased settings, and review selected bioengineering approaches which have been developed to replicate the properties of mineralized tissues and to initiate their repair.

Determinants of biomineralization

Mineral homeostasis

Homeostasis maintains key physiological variables such as ion concentrations, temperature and pH in an optimized range *in vivo*. The body is constantly interacting with the environment, however, and as a result, homeostasis *de facto* relies on a dynamic equilibrium provided by multiple control mechanisms and feedback loops. Moreover, with respect to ion homeostasis, there is a specific set point for each physiological milieu. Although the calcium concentration in serum is maintained by the endocrine system in a narrow range of 2.2-2.7 mmol/l[7], the intracellular calcium concentration is four orders of magnitude lower[3]. In bone, calcium ions are found as part of carbonated hydroxyapatite which belongs to the category of sparingly soluble minerals[8]. The solubility of ionic compounds is described by the solubility product constant, Ksp – the point of equilibrium between ionic, dissociated compounds and the undissolved solid. For carbonated hydroxyapatite at physiological conditions, the logarithm of Ksp is reported to be in the range of -58 – -59[9-11], whereas the logarithm of ionic product of calcium and phosphate in plasma is about -29 that is 30 orders of magnitude above the Ksp of carbonated hydroxyapatite. Yet, spontaneous precipitation of carbonated hydroxyapatite or its metastable precursors rarely happens in healthy soft tissues, unlike in bone, where in the latter case coordinated, cell-mediated processes of deposition and resorption are in dynamic equilibrium enabling the exchange of calcium and phosphate ions. It should be noted though that even in bone, carbonated hydroxyapatite does not nucleate directly, but rather results from a cascade that includes initially disordered, less stable precursors[12]. Even within small mineral particles a continuum of ordered and disordered phases can be observed[13], thus underscoring the dynamic equilibrium state of bone mineral. The discrepancies between the ion product and solubility, and the fine ionic homeostasis outside and within cells, highlight two paradoxes of biomineralization (FIG. 1). Firstly, the ability to confine carbonated hydroxyapatite formation to skeletal and dental tissues, and to avoid it (unless in pathology) in soft tissues. Secondly, the ability of the cells that directly facilitate appropriate formation of mineral to evade the toxic effects of high calcium and phosphate concentrations.

Throughout the body, an astonishing variety of tissues are based on a common building block – the collagen fibril. Collagen fibrils constitute a major component of the so-called connective tissues (which includes the specialized skeletal and dental connective tissues that mineralize). The fibrils are formed from triple helices stabilized by intermolecular hydrogen bonds. The fine fibrillar nature of collagen allows the lateral self-assembly and the formation of a staggered arrangement to give extensive arrays of collagen fibrils[14] which are further stabilised by covalent crosslinking[15, 16]. Enzymatic crosslinking of collagen is initiated by lysyl oxidase – a copper-dependent enzyme – resulting in precisely positioned aromatic bonds, namely one per collagen chain[17]. The complexity and hierarchical organization of collagen is tissue-specific[18, 19], as is the crosslinking (more specifically, the chemical nature and specificity of crosslinking and the combination of different types of collagens)[17, 20].

Biomineralization has evolved to diversify the mechanical properties of connective tissues. For example, the type I collagen fibrils of the dermis are not destined for mineralization to keep the skin compliant, whereas a similar collagen-based matrix must be mineralized in bones and teeth to render them rigid. Importantly, in bone, the extent of mineralization is maintained within a narrow range and is normally far from being uniform[18]. Interestingly, in some anatomical locations, such as at tendon attachment sites to bone or in the supporting tissues of a tooth, the interface between hard and soft tissues must be precisely graded for successful physiological performance. Thus, at hard-soft tissue interfaces, not only the extent of mineralization but also its submicron spatial gradients have to be precisely moderated.

General inhibition, selective promotion and selective inhibition

In a simplified way, regulation of biomineralization can be viewed as a hierarchical cascade of events where initially, abundant calcium and phosphate ions of the extracellular milieu are prevented from precipitation by systemic biomineralization inhibitors but in skeletal tissues the inhibitory mechanisms are annulled by the action of local biomineralization promoters (typically enzymes). Additionally, local biomineralization inhibitors are active in soft tissues that are at a higher risk of pathological mineralization (for example, cardiac valves, arteries and articular cartilage) and at hard-soft tissue interfaces (for example, tendon/ligament-bone attachments, cranial sutures and the periodontal ligament).

Pyrophosphate (PPi) and the protein fetuin A are examples of systemic inhibitors of calcium-phosphate precipitation from body fluids. PPi is a ubiquitous functional antagonist of inorganic phosphate (Pi), and an increase in the PPi/Pi ratio prevents spontaneous precipitation of mineral[21]. Interestingly, PPi concentrations maintained by osteoblasts in forming bone constitute an extracellular depot of compartmentalized phosphate until the PPi is actively enzymatically cleaved. Extracellular polyphosphate (polyPi, an extended analogue of PPi) also serves in the transport and compartmentalization of phosphates[22]. In the same way that glucose concentration is controlled by formation and destruction of glycogen, Pi concentration is regulated by formation and destruction of polyPi – a substrate for enzymatic cleavage in the skeleton[23]. PolyPi-packed granules chelate calcium ions, forming neutrally charged and amorphous complexes[23]; thus it seems that calcium-binding PPi and polyPi are a bioreservoir of mineral ions and effectively preclude collagen-based soft tissues from undesired mineralization.

The systemic mineralization inhibitor fetuin A is a circulating protein that in blood prevents the growth of nascent crystal nuclei and facilitates mineral particle recycling by macrophages. A single molecule of fetuin A can sequester up to 90-120 calcium atoms and 54-72 phosphate ions[24]. Fetuin A with calcium and phosphate ions forms colloidal complexes (calciprotein particles) about 30-150 nm in size[25]. Fetuin A has a strong affinity for bone tissue and comprises up to 25% of the noncollagenous proteins of bone[25].

As mineralization is central to skeletal function, to abolish the effect of biomineralization inhibitors in bone, osteoblasts express high levels of the PPi-degrading enzyme TNAP (tissue-nonspecific alkaline phosphatase)[26]. The enzymatic activity of TNAP on PPi not only removes a potent inhibitor of mineralization, but has the added advantage of simultaneously generating phosphate ions, thus in turn promoting mineralization. In soft tissues not destined for mineralization, the ratio of PPi/Pi remains high to inhibit mineralization. It has been suggested that polyPi granules contain alkaline phosphatase and other proteins; the notion here being that if alkaline phosphatase is activated, hydroxyapatite nucleates within the granule, displaces the protein component to the granule surface to result in the formation of a crystalline core coated with an amorphous shell[23].

The biomineralization promoter bone sialoprotein (BSP) is a calcium-binding SIBLING protein [Small Integrin-Binding Ligand N-linked Glycoprotein], the function of which appears to be mineral nucleation. It is present in the extracellular matrices of bone and teeth, but not in nonmineralized tissues[27]. Dentin matrix protein (DMP1) likewise is important in promoting extracellular matrix mineralization, as shown by mouse models and patients with deletion/mutations in the gene encoding DMP1 having autosomal recessive hypophosphatemic rickets (ARHR) characterized by osteomalacia. Mineralization promotion by the formation of mineral-protein complexes likely involves the stabilization of disordered mineral precursors by negatively charged proteins which localize to collagen fibril microdomains where the potential energy is lowest[28]. At these sites, the mineral component of the complex electrostatically interacts with collagen to influence fibril mineralization. At low concentrations of collagen reconstituted *in vitro*, the presence of negatively charged noncollagenous proteins can lead to *in vivo*-like intrafibrillar mineralization; whereas their absence results in the precipitation of extrafibrillar mineral globules[28]. However, at higher tissue-like densities of collagen fibrils, where dense monodispersed fibrils display cholesteric 3D alignment, the formation of intrafibrillar mineral has been shown to be possible without the involvement of noncollagenous proteins, and the morphology of the forming mineral reflects the spatial constraints of the dense collagen matrix[29]. As a consequence, the importance of mineral nucleators may well depend on the tissue suprafibrillar hierarchical architecture.

Locally in the extracellular matrix, mineralization-inhibiting proteins like osteopontin (OPN, abundant in bone) and matrix Gla protein (MGP, abundant in blood vessels and cartilage) either regulate crystal growth in the skeleton and dentition to fine-tune the overall favourable mineralization, or inhibit crystal growth completely. In the extracellular matrix of bone, nascent mineralization foci are always associated with OPN, which is subsequently found throughout mineralized bone matrix[30]. OPN also plays an important role in bone physiology as an interfacial protein bridging new bone to old bone (for example, at the cement lines located between osteons of different generations) and bridging new bone to implant surfaces[31]. OPN is present at interfaces where mineralization has to be abruptly quenched – another example being at tendon insertions to bone (entheses) or the periodontal ligament[32]. OPN-knockout mice do not exhibit macroscopic skeletal abnormalities (redundancy for regulating mineralization appears to be provided by other SIBLING proteins), but the size of the mineral crystals and their perfection are higher, indicating a lesser crystal growth restriction[33, 34], as would be expected given the loss of inhibitory OPN.

Although mineralization is systemically inhibited at most soft-tissue locations, articular cartilage and arteries appear to be at high risk for ectopic mineralization[35, 36]. The consequence of this is especially detrimental for their physiological function, and thus an additional line of defence against mineralization is required. Both chondrocytes and vascular smooth muscle cells express MGP and this is incorporated into their extracellular matrix. Defects in the gene encoding MGP in humans (causing Singleton-Merten syndrome, Keutel syndrome) lead to

catastrophic mineralization that causes fatal rupture of the aorta and/or premature growth plate fusion in long bones, and joint ankylosis[35]. For other mineralization inhibitors, such as Matrix Extracellular Phosphoglycoprotein (MEPE) and the peptide Acidic Serine and Aspartate-Rich Motif (ASARM) found in SIBLING proteins, the reader can be referred to excellent recent reviews[27, 37-39].

Compartmentalization of calcium and phosphate ions

Reports of the presence of vesicles in the extracellular matrix and of amorphous calcium phosphate mineral in developing bone, started to emerge nearly half-a-century ago[41, 42]. Whereas the plasma calcium level is high, intracellular calcium is kept low by sequestration by cytosolic organelles and proteins, yet calcium needs to be labile for its signalling role[3]. Protective mechanisms are thus in place by which cells can urgently decrease dangerously high levels of cytosolic calcium[3] by the budding off of calcium-rich vesicles into the extracellular matrix. As a result of calcium sequestration by proteins, PPi and polyPi, and following Ostwald's rule of phase transformation (in an environment of high saturation the first forming phase is likely to be metastable), extracellular vesicles contain disordered calcium phosphate[43]. Of note, that biomineralization proceeds via an amorphous pathway is well recognized in the realm of invertebrates who produce their skeletons of calcium carbonate[44].

More recently, high-resolution imaging of native frozen-hydrated specimens of developing bone tissue by cryo-electron microscopy with elemental analysis have confirmed the localization of amorphous calcium phosphate within intracellular vesicles and extracellular vesicles[45]. Despite the high elemental density of the vesicles loaded with disordered mineral, their Ca/Pi ratio was lower than that found in mature bone mineral and in stoichiometric hydroxyapatite. A feasible explanation is that the phosphate-rich phase (possibly polyPi) forms first and sequesters intracellular calcium which is otherwise toxic to cells[43]. An *in vitro* study of an osteoblastic culture showed that analogous intracellular granules containing amorphous calcium phosphate are associated with mitochondria[46]. Of note, in an *in vivo* study of developing bone in a zebrafish model using fluorescent confocal microscopy and Raman imaging, a nucleotide-like compound was also identified within the amorphous calcium phosphate-rich extracellular vesicles[47]. This is in line with observations that adenosine triphosphate (ATP) is an effective stabilizer of disordered calcium phosphate mineral[41]. Moreover, ATP is a substrate for TNAP[26] that is highly expressed by bone cells and not only cleaves phosphate to provide ions for mineralization, but destabilizes the amorphous phase and promotes the formation of nanocrystals within the collagenous template of bone. There is a possibility that amorphous mineral transport in vesicles is not only limited to bone cells and extracellular matrix, but also takes place in the peripheral bloodstream[47],[48].

Biomechanical implications of inhibition and promotion of mineralization

Most mineralization inhibitors, that may play a dual role as mineral nucleators under certain circumstances[27], are abundant in physiologically mineralized tissues. Redundant inhibitors are incorporated into bone for a strategic reason – mineralization has to be confined to a narrow range of about 65-70% (weight)[49]. As bone stiffness is inversely proportional to toughness and directly correlates with mineral content, uncontrolled mineralization can reduce work-to-fracture in bone[50-52]. There are also advantages to the properties of a biological material being predictable throughout the whole structure.

Heteronucleation – a process of the simultaneous formation of numerous crystallites in a chemically impure and biologically crowded milieu – is the most likely scenario in collagenous tissue biomineralization[41], where multiple nucleation sites are present within the extracellular matrix. However, matrix vesicles, the membrane of which differs somewhat from the cellular

membrane in its composition, provide a potential for significantly more abundant nucleation sites/promoters in the form of proteins and lipids, especially acidic phospholipids[53], in combination with their metastable ionic cargo and TNAP. As a result, the bone extracellular matrix is a polycrystalline composite, in which billions of nanocrystals have astonishingly reproducible habit, size and purity. Carbonated hydroxyapatite crystals in bone are reported to be plate-shaped, as opposed to being hexagonal/needle-shaped as they are in geogenic apatites[54], and crystal size in healthy bone does not exceed *ca*. 50 nm in the longest dimension[55, 56]. Finally, from an engineering point of view, bone hydroxyapatite is of 'low quality' attributable mainly to its many ionic and organic inclusions, substitutions and other imperfections[8].

Some studies on bone mineral structure have indicated that although the core of particles is crystalline, the periphery is highly substituted and practically amorphous [13, 23 57]. It has been demonstrated that structuring water is trapped as a rigid layer in the disordered outer shell of mineral crystallites and facilitates their co-oriented stacking at an additional hierarchical level[57]. Although amorphous calcium phosphate particles are transient in nature, stable amorphous calcium phosphate hydrophilic coatings on carbonated hydroxyapatite are present even in mature bone[57]. Thus, the structuring role of water is as important within the inorganic phase of skeletal tissues, as it is in the architecture of organic moieties: structural water imparts to bone mineral certain unique properties, such as the nano-size and the poor crystallinity[57]. From a physico-chemical aspect, the nanoscale size of the crystallites and their low purity, to a limited extent, compensate for the sparingly soluble nature of carbonated hydroxyapatite. From a biological perspective, the implications of the small, impure crystals are the following: high reactivity (high surface-to-bulk ratio, excessive ions can be sequestered or lacking ions can be mobilized), compatibility with the organic matrix (they can fit within and between crosslinked collagen fibrils without disrupting matrix integrity), and multiple interfaces are established at the nanoscale that hinder crack propagation. Another benefit of having small (rather than large) crystalline particles is that smaller stiff and brittle elements are less sensitive to defects and stress concentrators[58].

The small size of hydroxyapatite crystals lodged within a continuous collagenous framework allows for implementation of one of nature's most elegant biomechanical solutions – pre-stress[59, 60]. The hydration shell of collagen is provided by small proteoglycans SLRPs (Small Leucine-Rich Proteoglycans) that decorate the surface of collagen fibrils[61]. The precisely located, enzymatic covalent crosslinks in the collagenous scaffold confine bound water to the interfibrillar space[62]. As a result of the crosslinking, the collagenous matrix is effectively a continuous framework with limited extensibility that cannot swell to accommodate bound water, and the osmotic pressure provided by proteoglycans builds up to the kPa range[63-65]. During the process of mineral nucleation and growth in bone, water is gradually displaced by the crystallites[66]. Hence, the collagen matrix imparts compressive stress on the mineral phase developed under the spatial constraints of the aligned and crosslinked framework[63]. The net result of putting the collagenous scaffold under pre-tension, and the mineral component under pre-compression, is a toughening of the structure. Pre-tension ensures collagen fibrils remain in tension under most physiological strains, including under compression, as compressive stress will first be dissipated on pre-tension neutralization[67]. The same holds true for the hydroxyapatite crystals; they are exceptionally resistant to compression, a degree of pre-compression (0.08%[67]) will defer the critical tensile loads. In summary, both the organic and inorganic constituents synergistically work in their preferred mode by virtue of their pre-stressed structural arrangement. Amongst synthetic materials, the closest analogue is reinforced concrete, in which rods of ductile steel are under tension, and stiff concrete is under compression.

The noncollagenous proteins intercalated with the stress-bearing collagenous framework serve as a basis for an important biomechanical phenomenon – sacrificial bonds. Sacrificial bonds bridge dissimilar constituents in a composite[68]; they can be severed at critical loads, but then re-established at rest[50, 69]. Sacrificial bonds provide the first tier in bearing high stress. Further stress is absorbed by the collagen chains themselves, with only the highest stress being imparted to the mineral crystallites[69]. Indeed, as found by simultaneous monitoring of strain in bone by a strain gauge, small-angle scattering and wide-angle diffraction, the strains experienced by the macro-specimen, its collagen framework and the crystals, are not equal. Of the net strain experienced by the specimen, only 80% is effectively transduced to collagen fibrils, meaning that before they are loaded there is a limited amount of shear between adjacent fibrils. In turn, of the net strain experienced by collagen fibrils, only about 90% is transduced onto the hydroxyapatite crystallites, with the remainder being absorbed by the interfacial proteins[69, 70].

The microheterogeneity of bone is an important toughening mechanism, which likely also defines the difference between the properties of young and senescent bone. Most of a mature skeleton is comprised of lamellar bone, which can be thought of as a plywood-like layering of collagen arrays oriented in different directions. In fact, within lamellar bone, 10-15% of collagen fibrils form disordered arrays[71, 72]. These disordered arrays are positioned at the boundaries of 2-3 micrometre-thick ordered lamellae with distinct orientation of collagen fibrils. The disordered layers at the interlamellar boundaries are less mineralized, in addition to the loose packing of collagen and higher hydration[73]. This contributes to microscale fluctuations of the Young's modulus; the numerous interfaces of varying stiffnesses, together with alternating collagen alignment, explain the observation that a crack in healthy bone never follows a straight line (an energetically low-cost path). Deviation of the crack leads to energy dissipation and is visible as a 'zig-zag' pattern at the fracture surface[50].

Cardiovascular mechanics

The cardiovascular system is the second most mechanically challenged system after the musculoskeletal system (Box 1). Loading of the heart and arteries is automatic and repetitive (FIG. 2), driven by the pace-making apparatus of the beating heart and moderated by autonomous nervous signals and adreno-cortical humoral signals. The life-long provision of steady orthograde blood flow, together with the attenuation of the pulsatile waves in the peripheral tissues, put a special set of mechanical requirements on the components of the cardiovascular system: namely, compliance, resilience and fatigue-resistance.

With ageing, the cardiac valves and the arterial walls become more rigid with declining capacity to ensure one-way blood flow and to dampen pulsatile waves. The higher rigidity of aged valves and arteries can be attributed to reduced collagen compliance and impaired elastin recoil. With gradual cardiovascular stiffening often comes mineralization – a qualitative transition whereby a compliant organic structure accrues an abnormal and debilitating inorganic constituent. There are four principal types of cardiovascular mineralization: atherosclerotic calcification, medial artery calcification, cardiac valve calcification and vascular calciphylaxis. These types of calcificaion can co-exist, escalating the severity of a clinical condition[74]. Although calcification in atherosclerosis can be viewed as a compensatory response to a chronic inflammation (as observed in tuberculosis lesions, carcinomas, and parasite invasion sites), and vascular calciphylaxis is an extreme example of metabolic disorder with up to 80% mortality rate[74], in this Review, we mainly focus on medial and valvular calcification (Table 1).

Cardiovascular mineralization

To understand the pathophysiology of cardiovascular calcification, one must appreciate the balance between the local anti- and pro-mineralization factors acting in a dynamic and mechanically challenged milieu[75-77]. The pro-mineralization agents include ubiquitous calcium and phosphate ions, and a collagen- and elastin-rich tissue framework. These are opposed by anti-mineralization factors, both systemic (PPi and fetuin A)[25] and local (MGP)[35]. Local nucleators of mineralization, such as thrombi, cell debris, matrix vesicles and activated platelets may favour calcium phosphate mineral nucleation upon local trauma or necrosis[78]. Of note, injury to the intima triggers OPN expression by vascular smooth muscle cells in the arterial media; thus, OPN, which is normally absent in the arterial wall, may appear as a second line of defence against unwanted local calcification[79].

Cardiovascular mineralization occurs systemically if the pro- and anti-mineralization factors are not balanced, as in the example of the genetic loss of MGP function (Singleton-Merten[80] and Keutel[81] syndromes), or as in the case of an abnormal concentration of circulating phosphate in uraemia[82]. An elevated plasma phosphate level readily leads to the formation of ectopic calcification foci in various soft tissues (skin, kidneys, tendons), but is most significant in arteries, namely in the *tunica media*. Medial arterial calcification, can occur not only in uraemia, but may also present in type 2 diabetes and aggravates the prognosis for both[83].

Intriguingly, we have reported that highly-crystalline spherical mineral particles can be found in both the aortic and valvular tissue not manifesting any macroscopic calcification, and also within calcific lesions in pathological vascular tissue and cardiac valves[84]. In some of the macroscopic calcification sites it was possible to identify these particles either associated with mineralized fibres or embedded within compact calcified areas[84]. The origin of the spherical mineral particles remains of great interest; in particular, with regards to their high crystallinity, their spherical shape and their lack of a structural association with extracellular matrix fibres. Unveiling the full mechanism of pathological cardiovascular calcification is thus an active are of research and many questions remain on the origin of the mineral and its nucleation and growth. It is important to note that the cardiovascular calcification comprises a different variety of calcium phosphate to bone (whitlockite versus hydroxyapatite[85]) and cardiovascular mineralization is thus quite different from bone proper. Nonetheless, a postulated driving force for cardiovascular calcification at the local level is provided by osteoblasts-like cells, which can transdifferentiate from local pericytes and smooth muscle cells, or can differentiate from circulating pluripotent cells[74]. Vascular smooth muscle cells display signs of transdifferentiation towards chondro/osteoprogenitors, and their transcription profile ultimately overlaps with that of osteoblasts (although it does not fully match). What is less clear, however, is whether mineralization precedes this cellular change[86], or whether it follows it[87]. The chondro/osteogenic shift of the transcription profile is associated with pro-inflammatory factors such as TNFα and vascular calcification in general is accompanied by neoangiogenesis[74]. It is conceivable that the inflammatory component is more characteristic of local cardiovascular calcification primarily associated with cell transdifferentiation[87], whereas systemic vascular calcification is rather induced, independently of inflammation by metabolic imbalance and alteration of the ionic equilibrium[83]. Although atherosclerosis-associated vascular calcification merits a separate review, it is intriguing from the biomechanical perspective that a higher heterogeneity of a calcifying plaque, and the presence of numerous interfaces between its organic and inorganic constituents, lead to plaque instability and higher risk of disintegration followed by thrombosis[88]. In contrast, macrocalcification of the plaque sustains plaque integrity and bears a lesser risk of thrombosis[88]. To compare to physiological mineralization, in bone

the presence of abundant interfaces with abruptly changing moduli is essential for adequate toughness[50].

Cells are generally responsive to the stiffness of the extracellular matrix—to such an extent that the fate of stem cells is strongly influenced by the substrate's Young's modulus[89]. It seems reasonable that abnormal stiffening of the substrate might contribute to the acceleration of osteogenic transdifferentiation and mineralization[86]. The effect of the substrate stiffness is not the only factor governing cell fate, but is likely synergistically enhanced by the differentiation potential ('stemness') of the cells in question. Indeed, injection of mesenchymal stem cells into relatively stiff infarcted heart tissue in mice led to the eventual formation of calcific lesions, possibly occurring through differentiation of the cells towards the osteogenic lineage[90]. In contrast, injected fibroblasts or hematopoietic progenitors in the same mouse model were not associated with cardiac tissue calcification. Nevertheless, tissue stiffness may also be increased by the accumulation of nonspecific crosslinks in the collagen framework induced by advanced glycation end-products (AGE). This nonenzymatic reaction between sugar metabolites results in predominantly protein crosslinking (but also in lipid and nucleic acid crosslinking), and increases tissue rigidity (Table 2). Although primarily attributed to uncompensated diabetes, this phenomenon is in fact associated with ageing in general, and largely affects tissues with a slow turnover rate. As lipids are also a substrate for AGE, nonenzymatic crosslinking aggravates atherosclerotic plaque instability. Thus, nonspecific AGE crosslinking of collagen, elastin and lipids could explain the higher incidence of both atherosclerotic (intimal) and nonatherosclerotic (medial) vascular calcification in the elderly, attributable to their generally slower metabolism. In fact, the devastating effect of AGE-initiated nonenzymatic crosslinking can be found throughout all connective tissues, including bone, cartilage, dermis, the ocular lens capsule, blood vessels and cardiac valves, and is inevitably associated with age and amplified by glycaemia (either diabetic or dietary) and impaired renal clearance.

Cardiovascular prostheses composed of processed cadaveric or xenogeneic tissue are commonly used to replace defective cardiac valves or arteries. These bioprostheses possess excellent hydrodynamic behaviour and low thrombogenicity. Glutaraldehyde crosslinking is commonly used for preservation, fixation and sterilization of the bioprosthetic tissue, which would otherwise evoke an inflammatory foreign body reaction, followed by resorption of the graft by the host[91]. However, aldehyde-treated tissues possess inferior resilience upon cycling loading[92] and also eventually deteriorate and rupture following pathological calcification. Interestingly, glutaraldehyde fixation significantly stiffens the tissue and reduces its stress relaxation rate. In the sense of being a nonspecific tissue stiffener, the glutaraldehyde effect resembles that of AGE (Table 2). A promising research direction on cardiovascular grafts is the evaluation of alternative fixation methods that would allow the maximal conservation of the biomechanical properties of a tissue, and defer the calcification events. For example, the use of a carbodiimide-based compound has been reported as a crosslinking agent that results in better biocompatibility and minimal calcification of a transplant in the short term[93]. Genipin, an alternative crosslinking agent that is applied for collagen hydrogels[94], shows encouraging results in cardiovascular transplant processing in animal models[95].

Engineering control of biomineralization

Inspiration from nature's strategies of biomineralization control can be taken and applied to the field of artificially engineered materials. Take, for example, the use of biomolecules for inhibition or for nucleation of mineral. It is noteworthy that phosphate and calcium behave in radically different ways in binding to biomolecules. Although phosphate can be polymerized

or covalently attached to polymers, calcium ions bind electrostatically to negatively charged polymers. Hence, phosphate metabolism requires the action of enzymes, such as TNAP, and calcium metabolism is controlled by pH and salinity of the solution. Given that both calcium and phosphate are needed for mineral formation, this provides an interesting flexibility in controlling the mineralization process. In order to regulate the phosphate contribution to biomineralization, using TNAP enzyme control has proven to be a useful lever. In both the case of healthy skeletal development and in the deterioration of an aldehyde-treated bioprosthetic cardiac valve, phosphate ions are released by TNAP and precipitate together with calcium on the collagenous framework; yet in one case the effect is beneficial[96], and in the other case it is detrimental[91, 97] (Table 1). Thus by intervening and moderating TNAP activity, for example by addition of $AlCl_3$ or $FeCl_3$ salts to transplanted valves to block TNAP activity, one can delay the onset of valve calcification[97]. Conversely, delivery of active TNAP as an enzyme-replacement therapy has recently brought to clinical practice a treatment for hypophosphatasia (HPP) to promote bone mineralization[98]. Indeed, hypophosphatasia results from loss-of-function mutations in the gene encoding TNAP such that the mineralization-inhibiting substrate (PPi) for this enzyme accumulates in skeletal and dental tissues, causing osteomalacia, odontomalacia, hypercalcaemic seizures and even commonly death in infancy from respiratory insufficiency (due to a soft hypomineralized rib cage). In hypophosphatasia, the extracellular matrix collagenous framework of bone and the inorganic ions are indeed available, but biomineralization is hindered because of excessive local inhibitory PPi. In the enzyme-replacement therapy, recombinant TNAP is targeted to residual skeletal and tooth mineral in the patients via a mineral-binding deca-aspartate peptide sequence, a delivery method that contributes to the rescue of the skeletal and dental mineralization defect[98, 99]. Targeting of the TNAP precisely to residual bone and tooth mineral because of the presence of the mineral-binding peptide prevents such possible adverse effects as unwanted mineralization of soft tissues at the site of injection (the skin), or more distally after its uptake in the circulation[99]. However, the relative contributions to the success of this patient treatment of the mineral-targeted enzyme versus the circulating enzyme remains to be determined.

TNAP can also be immobilized to induce mineralization onto the surface of biomaterials meant to replace defective bone. For example, the coupling of TNAP to a chitosan matrix favours cell adhesion and results in increased scaffold toughness attributable to localized mineralization, as validated by X-ray diffraction analysis[100].

Due to the potential electrostatic interactions of calcium, either a pro- or anti-mineralization milieu can be artificially created for use in biomaterials development by judicious selection of appropriately charged substrates (for example, polymers, peptides, and recombinant proteins). Furthermore, collagenous matrices can be pre-mineralized by applying concentrated solutions of calcium and phosphate ions (for example, using simulated body fluid), and then used as osteoinductive scaffolds that promote osteoblast binding[101,102]. However, achieving the fine intrafibrillar mineralization found in bone, as discussed above, requires important regulatory determinants besides the calcium-phosphate product and the available collagenous framework. Intrafibrillar collagen mineralization has been achieved *in vitro* by adding polyaspartate peptide to a neutral solution of calcium and phosphate in which a highly hydrated amorphous-phase mineral is stabilized as a polymer-induced liquid precursor (PILP)[103]. The stabilized amorphous mineral then infiltrates collagen fibrils and crystallizes therein, thus forming a hierarchical structure. This process when applied to either demineralized bone or reconstituted collagen fibrils produces intrafibrillar, aligned nanocrystalline hydroxyapatite[104].

Even in the absence of molecular or ionic biomimetic cues, biomaterial-induced mineralization can be tailored through changes in biomaterial properties such as topography and stiffness.

The stiffness of the substrate, as mentioned above, has been shown to influence whether mesenchymal stem cells take a neurogenic or osteogenic differentiation route, with the latter being favoured in the presence of a higher Young's modulus of 25-40 kPa versus 1 kPa[89]. Furthermore, even small changes in surface roughness[105, 106], or the order/disorder of nanoscale pits on a substrate, can strongly influence mineralization[107]. The strongest osteogenic commitment was reported for a culture grown on a substrate with a surface roughness *ca*. 2-3 µm and the distance between micro-asperities 50-70 µm (measured by alkaline phosphatase activity and collagen I gene expression)[108]. However, in other cell evaluation experiments, the effect of surface roughness levels off as a culture develops and matures[109], perhaps attributable to deposition of the ECM by the cell culture and masking of the original surface roughness. Currently, scaffolds composed of functionalised materials can be custom-built from an array of techniques to suit the size and type of defect, as well as the regenerative capacity of the patient. Ideally, these scaffolds should aim to combine the macroscopic geometry and stiffness required to support loads, with meso- and cell-scale structures, stiffnesses, and materials that take into consideration pro- or anti-mineralization cues as required for a particular application.

Conclusions and future perspectives

Nature has created a redundancy of control mechanisms which together make the biomineralization cascade predictable and finely regulated, both temporarily and spatially. This elegant machinery involves the simultaneous action of inhibitors and promoters, and enzyme-enabled amplification loops. However, this versatility in the complex regulatory control of biomineralization comes at a price – a failure in just one nexus in this chain of regulation may have life-threatening consequences, as seen in individual genetic disorders such as osteogenesis imperfecta and vitamin D-resistant rickets. Although these genetic conditions are rare, the intricate biomineralization control mechanisms that have evolved over hundreds of millions years for hardening bones and teeth, and for preventing the debilitating calcification of soft tissues in the cardiovascular system, are seemingly unable to keep pace with recent changes in human lifestyle, particularly our diet and our increasing longevity[110].

The first known prosthetic replacement of a body part (a right big toe) took place around four thousand years ago in ancient Egypt[111]. The carved wooden toe had wear patterns indicating that it was functional for years. The desire to recreate missing or damaged tissues is even more pressing today, with a shift from using inert prosthetics that simply replace the lost organ, to the use of cell-instructive biomaterials, which are capable of a far more intimate integration into the host by means of maximizing the regenerative potential of the cells. The ultimate goal of 'cell-instructive biomaterials' could be seen in complete regeneration of tissues and organs achieved by appropriate tuning of the host cellular responses using ionic dopants, signalling molecules or peptides, in combination with physical cues (for example substrate roughness, stiffness, curvature).

As the materials analysis field advances, one can gain even greater insight into the intricate structure of natural materials. For example, high-resolution electron microscopy imaging used today in the field of structural biology cannot be even thought of without the use of advanced cryo-preservation methods to keep molecules and cells in their pristine, hydrated native state, as opposed to the chemical fixation that is associated with dehydration and distortion of the sample[43, 112, 113]. Biomineralization is a multi-step process involving mineral-protein complexes and meta-stable compounds (such as amorphous calcium-phosphate), and therefore, arguably the most informative way to interrogate this in detail might be dynamic liquid-cell or atmospheric electron microscopy to provide analysis under more native aqueous conditions[113,

[114]. As another example, Raman spectroscopy – originally developed for material characterization – has been successfully adapted for *in situ* monitoring of mineralization in living cells and tissues *in vivo* and *in vitro*[115]. In this way, advances in state-of-the-art technology can unravel the mysteries of nature and likewise provide tools for biomimetic design. As the resolution of modern imaging methods constantly improves, it is important to keep findings contextualized and not to lose track of the bigger picture. This is especially important for imaging of hierarchically organized biological materials and in accounting for normal biological variation where correlative imaging is gaining momentum. By combining two, three or even more imaging methods that allow co-localization of a feature of interest, the resolution and the volume of imaging can span 7-8 orders of magnitude (FIG. 3 and Table 3).[115-129]

The principles governing the mineralization of biological tissues, or those preventing it from mineralizing, have evolved over millions of years into robust mechanisms that function under a variety of environmental challenges. A detailed understanding of such principles is naturally inspiring new approaches in engineering. This includes self-organization processes and mimicking the multi-scale hierarchical structure of mineralized tissues. However, many processes are poorly understood and, in particular, the dynamics of mineral (ion) transport, intermediate storage and on-time delivery remain a mystery in most mineralizing tissues. Moreover, mechanosensitivity of cells integrates mechanical stimuli into growth and adaptation of skeletal tissues. This raises a number of important research questions, some examples are summarized in Figure 4. These questions need to be addressed if we want to push our knowledge from a simple description of structures and their material properties to a more complete understanding of how these structures are formed, repaired and adapted to changing needs. Such understanding will also improve our abilities to conceive new types of active, adaptive and self-repairing materials. Finally, there is a counter-intuitive character of many of nature's technological solutions – namely, that imperfections (in their common connotation) can be biologically advantageous in several different ways[130]. Skeletal fragility, for example, is reduced by the inhomogeneity of bone, while the biological activity of mineral crystals results from their imperfection, and mechanosensation of bone cells is facilitated by local disorder in bone collagen organization. The intentional productive use of imperfection is a hallmark of materials engineering, making biomineralization particularly inspiring for discoveries in materials science.

[131, 132]

Conflicts of interest

The authors declare no conflict of interests.

Acknowledgements

We thank Professor Marc McKee, McGill University, for critical reading of this manuscript and insightful comments.

References

1. Pearce, P. Structure in Nature Is a Strategy for Design (MIT Press, Boston, MA, USA, 1990).

2. Thompson, D.W. On Growth and Form (Cambridge University Press, Cambridge, 1942).

3. Campbell, A.K. Calcium as an intracellular regulator. *Proceedings of the Nutrition Society* **49**, 51-56 (1990).

4. Penido, M.G.M.G., Alon, U.S. Phosphate homeostasis and its role in bone health. *Pediatr Nephrol* **27**, 2039-2048 (2012).

5. Hunter, G.K., Kyle, C.L. & Goldberg, H.A. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J* **300**, 723-728 (1994).

6. Addison, W.N., Masica, D.L., Gray, J.J. & McKee, M.D. Phosphorylation-dependent inhibition of mineralization by osteopontin ASARM peptides is regulated by PHEX cleavage. *J Bone Miner Res* **25**, 695-705 (2010).

7. Goldstein, D.A. Clinical Methods: The History, Physical, and Laboratory Examinations (Butterworths, Boston, MA, USA, 1990).

8. Magalhaes, M.C.F., Marques, P.A.A.P., Correira, R.N. Biomineralization. Medical Aspects of Solubility (ed. Koenigsberger, E., Koenigsberger, L.) (John Wiley and Sons Ltd., Chichester, UK, 2006).

9. Moreno, E.C., Gregory, T.M., Brown, W.E. Preparation and solubility of hydroxyapatite. *Journal of Research of the National Bureau of Standards - A. Physics and Chemistry* **72A**, 773-782 (1968).

10. Elliott, J.C. Calcium phosphate biominerals. *Rev. Geochem. Mineral.* **48**, 427-453 (2002).

11. Elliott, J.C. Hydroxyapatites and nonstoichiometric apatites. *Studies in Inorganic Chemistry* **18**, 111-189 (1994).

12. Habraken, W.J.E.M., Tao, J., Brylka, L.J., Friedrich, H., Bertinetti, L., Schenk, A.S., Verch, A., Dmitrovic, V., Bomans, P.H.H., Frederik, P.M., Laven, J., van der Schoot, P., Aichmayer, B., de With, G., De Yoreo, J.J., Sommerdijk, N.A.J.M. Ion-association complexes unite classical and non-classical theories for the biomimetic nucleation of calcium phosphate. *Nature Communications* **4** (2013).

13. De Yoreo, J.J., Gilbert, P.U.P.A., Sommerdijk, N.A.J.M., Penn, R.L., Whitelam, S., Joester, D., Zhang, H., Rimer, J.D., Navrotsky, A., Banfield, J.F., Wallace, A.F., Michel, F.M., Meldrum, F.C., Coelfen, H., Dove, P.M. Crystallization by particle attachment in synthetic, biogenic, and geologic environments. *Science* **349**, aaa6760 (2015).

14. Hodge, A.J. & Petruska, J.A. in Aspects of Protein Structure (ed. Ramachandran, G.N.) 289-300 (Academic Press, New York, 1963).

15. Saito, M., Marumo, K. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. *Osteoporosis Int* **21**, 195-214 (2010).

16. Eyre, D.R., Dickson, I.R., Van Ness, K. Collagen cross-linking in human bone and articular cartilage. *Biochem J* **252**, 495-500 (1988).

17. Fratzl, P. Collagen: structure and mechanics (Springer Science + Business Media, LLC, New York, USA, 2008).

18. Fratzl, P., Weinkamer, R. Nature's hierarchical materials. *Progress in Materials Science* **52**, 1263-1334 (2007).

19. Reznikov, N., Shahar, R., Weiner, S. Bone hierarchical structure in three dimensions. *Acta Biomaterialia* **10**, 3815-2826 (2014).

20. Yamauchi, M., Chandler, G.S. & Katz, E.P. in Chemistry and Biology of Mineralized Tissues. (eds. Slavkin, H. & P., P.) 39-46 (Elsever Science Publishers, 1992).

21. Fleisch, H., Russell, R.G.G., Straumann, F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature* **5065**, 901-903 (1966).

22. Omelon, S. et al. Control of vertebrate skeletal mineralization by polyphosphates. *PLoS One* **4**, e5634 (2009).

23. Omelon, S., Ariganello, M., Bonucci, E., Grynpas, M., Nanci, A. A review of phosphate mineral nucleation in biology and geobiology. *Calcified Tissue International* **93**, 382-396 (2013).

24. Jahnen-Dechent, W., Schaefer, C., Ketteler, M., McKee, M.D. Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. *Journal of Molecular Medicine* **86**, 379-389 (2008).

25. Jahnen-Dechent, W., Heiss, A., Schaefer, C., Ketteler, M. Fetuin A regulation of calcified matrix metabolism. *Circulation Research* **108**, 1494-1509 (2011).

26. Millan, J.L. Alkaline phosphatases. Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signalling* **2**, 335-341 (2006).

27. George, A., Veis, A. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. *Chem Rev* **108**, 4670-4693 (2008).

28. Nudelman, F. et al. The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. *Nature Materials* **9**, 1004-1009 (2010).

29. Wang, Y., Azais, T., Robin, M., Vallee, A., Catania, C., Legriel, P., Pehau-Arnaudet, G., Babonneau, F., Giraud-Guille, M.-M., Nassif, N. The predominant role of collagen in the nucleation, growth, structure and orientation of bone apatite. *Nature Materials* **11**, 724-733 (2012).

30. McKee, M.D., Nanci, A. Osteopontin: an interfacial extracellular matrix protein in mineralized tissues. *Connect Tissue Res* **35**, 197-205 (1996).

31. McKee, M.D., Nanci, A. Osteopontin at mineralized tissue interfaces in bone, teeth and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover and repair. *Microscopy Reasearch and Technique* **33**, 141-164 (1996).

32. McKee, M.D., Zalzal, S., Nanci, A. Extracellular matrix in tooth cementum and mantle dentin: localization of osteopontin and other noncollagenous proteins, plasma proteins, and glycoconjugates by electron microscopy. *The Anatomical Record* **245**, 293-312 (1996).

33. Kavukcuoglu, N.B., Denhardt, D.T., Guzelsu, N. & Mann, A.B. Osteopontin deficiency and aging on nanomechanics of mouse bone. *J Biomed Mater Res A* **83**, 136-44 (2007).

34. Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B., McKee, M.D. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. *Calcified Tissue International* **71**, 145-154 (2002).

35. Luo, G., Ducy, P., McKee, M.D., Pinero, G.J., Loyer, E., Behringer, R.R., Karsenty, G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* **386**, 78-81 (1997).

36. Schinke, T., McKee, M.D., Karsenty, G. Extracellular matrix calcification: where is the action? *Nature Genetics* **21**, 150-151 (1999).

37. Gorski, J.P. Is all bone the same? Distinctive distributions and properties of non-collagenous matrix proteins in lamellar vs. woven bone imply the existence of different underlying osteogenic mechanisms. *Critical Reviews in Oral Biology and Medicine* **9**, 201-23 (1998).

38. Boskey, A.L. Biomineralization:conflicts, challenges and opportunities. *J. Cell. Biochem. Suppl.* **30-31**, 83-91 (1998).

39. Veis, A. in Biomineralization. Reviews in Mineralogy and Geochemistry (eds. Dove, P.M., De Yoreo, J.J. & Weiner, S.) 249-290 (Mineralogical Society of America, Washington DC, 2003).

40. Barros, N.M.T., Hoac, B., Neves, R.L., Addison, W.N., Assis, D.M., Murshed, M., Carmona, A.K., McKee, M.D. Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia. *Journal of Bone and Mineral Research* **28**, 688-699 (2013).

41. Posner, A.S., Betts, F., Blumenthal, N.C. Properties of nucleating systems. *Metabolic Bone Disease* **1**, 179-183 (1978).

42. Betts, F., Blumenthal, N.C., Posner, A.S. Bone mineralization. *Journal of Crystal Growth* **53**, 63-73 (1981).

43. Mahamid, J., Sharir, A., Gur, D., Zelzer, E., Addadi, L., Weiner, S. Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryo-electron microscopy study. *J Struct Biol* **174**, 527-535 (2011).

44. Politi, Y., Arad, T., Klein, E., Weiner, S. & Addadi, L. Sea urchin spine calcite forms via a transient amorphous calcium carbonate phase. *Science* **306**, 1161-4 (2004).

45. Mahamid, J., Sharir, A., Addadi, L., Weiner, S. Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: indications for an amorphous precursor phase. *PNAS* **105**, 12748-12753 (2008).

46. Boonrungsiman, S., Gentleman, E., Carzaniga, R., Evans, N.D., McComb, D.W., Porter, A.E., Stevens, M.M. The role of intracellular calcium phosphate in osteoblast-mediated bone apatite formation. *PNAS* **109**, 14170-14175 (2012).

47. Akiva, A., Malkinson, G., Masic, A., Kerschnitzki, M., Bennet, M., Fratzl, P., Addadi, L., Weiner, S., Yaniv, K. On the pathway of mineral deposition in larval zebrafish caudal fin bone. *Bone* **75**, 192-200 (2015).

48. Kerschnitzki, M., Akiva, A., Ben Shoham, A., Koifman, N., Shimoni, E., Rechav, K., Arraf, A.A., Schultheiss, T.M., Talmon, Y., Zelzer, E., Weiner, S., Addadi, L/. Transport of membrane-bound mineral particles in blood vessels during chicken embryonic bone development. *Bone* **83**, 65-72 (2016).

49. Currey, J.D. Bones: Structure and Mechanics (Princeton University press, Oxford, 2002).

50. Wagermaier, W., Klaushofer, K., Fratzl, P. Fragility of bone material controlled by internal interfaces. *Calcified Tissue International* **97**, 201-212 (2015).

51. Martin, R.B., Burr, D.B. & Sharkey, N.A. Skeletal Tissue Mechanics (Springer-Verlag, New York, 1998).

52. Helfrich, M.H. Osteoclast diseases. *Microscopy Research and Technique* **61**, 514-32 (2003).

53. Golub, E.E. Role of matrix vesicles in biomineralization. *Biochimica et Biophysica Acta* **1790**, 1592-1598 (2009).

54. Landis, W.J., Hodgens, K.J., Arena, J., Song, M.J. & McEwen, B.F. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. *Microsc. Res. Tech.* **33**, 192-202 (1996).

55. Weiner, S. & Price, P. Disaggregation of bone into crystals. *Calcif Tissue Int.* **39**, 365-375 (1986).

56. Moradian-Oldak, J., Weiner, S., Addadi, L., Landis, W.J. & Traub, W. Electron imaging and diffraction study of individual crystals of bone, mineralized tendon and synthetic carbonate apatite. *Connect Tissue Res* **25**, 219-28 (1991).

57. Wang, Y., Von Euw, S., Fernandes, F.M., Cassaignon, S., Selmane, M., Laurent, G., Pehau-Arnaudet, G., Coelho, C., Bonhomme-Coury, L., Giraud-Guille, M.-M., Babonneau, F., Azais, T., Nassif, N. Water-mediated structuring of bone apatite. *Nature Materials* **12**, 1144-1153 (2013).

58. Ennos, R. Solid biomechanics (Princeton University Press, Woodstock, England, 2012).

59. Wainwright, S.A., Biggs, W.D., Currey, J.D. & Gosline, J.M. Mechanical Design in Organisms (Princeton University Press, Princeton, 1976).

60. Gordon, J.E. Structures: or why things don't fall down (Da Capo Press, Reading, England, 2003).

61. Nikitovic, D., Aggelidakis, J., Young, M.F., Iozzo, R.V., Karamanos, N.K., Tzanakakis, G.N. The biology of small leucine-rich proteoglycans in bone pathophysiology. *Journal of Biological Chemistry* **287**, 33926-33933 (2012).

62. Allen, M.R., Newman, C.L., Chen, N., Granke, M., Nyman, J.S., Moe, S.M. Changes in skeletal collagen cross-links and matrix hydration in high- and low-turnover chronic kidney disease. *Osteoporosis Int* **26**, 977-985 (2015).

63. Bertinetti, L. et al. Osmotically driven tensile stress in collagen-based mineralized tissues. *J Mech Behav Biomed Mater* **52**, 14-21 (2015).

64. Masic, A., Berrtinetti, L., Schuetz, R., Chang, S.-W., Metzger, T.H., Buehler, M.J., Fratzl, P. Osmotic pressure induces tensile forces in tendon collagen. *Nature Communications*, 6:5942 (2015).

65. Maroudas, A. Balance between swelling pressure and collagen tension in normal and degenerate cartilage. *Nature* **260**, 808-809 (1976).

66. Timmins, P.A. & Wall, J.C. Bone water. *Calcified Tissue Research* **23**, 1-5 (1977).

67. Forien, J.B. et al. Compressive residual strains in mineral nanoparticles as a possible origin of enhanced crack resistance in human tooth dentin. *Nano Letters* **15**, 3729-3734 (2015).

68. Kaartinen, M.T., El-Maadawy, S., Rasanen, N.H., McKee, M.D. Tissue transglutaminase and its substrates in bone. *Journal of Bone and Mineral Research* **17**, 2161-2173 (2002).

69. Gupta, H.S., Seto, J., Wagermaier, W., Zaslansky, P., Boesecke, P., Fratzl, P. Cooperative deformation of mineral and collagen in bone at the nanoscale. *PNAS* **103**, 17741-17746 (2006).

70. Hoo, R.P., Fratzl, P., Daniels, J.E., Dunlop, J.W.C., Honkimaeki, V., Hoffmann, M. Cooperation of length scales and orientations in the deformation of bovine bone. *Acta Biomaterialia* **7**, 2943-2951 (2011).

71. Reznikov, N., Almany-Magal, R., Shahar, R., Weiner, S. Three-dimensional imaging of collagen fibril organization in rat circumferential lamellar bone using a dual beam electron microscope reveals ordered and disordered sub-lamellar structures. *Bone* **52**, 676-683 (2013).

72. Reznikov, N., Shahar, R., Weiner, S. Three-dimensional structure of human lamellar bone: the presence of two different materials and new insights into the hierarchical organization. *Bone* **59** (2014).

73. Utku, F.S., Klein, E., Saybasili, H., Yucesoy, C.A., Weiner, S. Probing the role of water in lamellar bone by dehydration in the environmental scanning electron microscope. *J Struct Biol* **162**, 361-367 (2008).

74. Johnson, R.C., Leopold, J.A., Loscalzo, J. Vascular calcification. Pathobiological mechanisms and clinical implications. *Circulation Research* **90**, 1044-1059 (2006).

75. Lee, D. Vascular calcification: Inducers and inhibitors. *Materials Science and Engineering B* **176**, 1133-1141 (2011).

76. Marulanda, J., Alqarni, S., Murshed, M. Mechanisms of vascular calcification and associated diseases. *Current Pharmaceutical Design* **20**, 1-10 (2014).

77. Ruiz, J.L., Hutcheson, J.D., Aikawa, E. Cardiovascular calcification: current controversies and novel concepts. *Cardiovascular Pathology* **24**, 207-212 (2015).

78. Clarke, M.C.H., Littlewood, T.D., Figg, N., Maguire, J.J., Davenport, A.P., Goddard, M., Bennett, M.R. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circulation Research* **102**, 1529-1538 (2008).

79. Liaw, L., Lindner, V., Schwartz, S.M., Chambers, A.F., Giachelli, C.M. Osteopontin and beta 3 integrin are coordinately expressed in regenerating endothelium in vivo and stimulate Arg-Gly-Asp-dependent endothelial migration in vitro. *Circulation Research* **77**, 665-672 (1995).

80. Singleton, E.B., Merten, D.F. An unusual syndrome of widened medullary cavities of the metacarpals and phalanges, aortic calcification and abnormal dentition. *Pediatric Radiology* **1**, 2-7 (1973).

81. Munroe, P.B., Olgunturk, R.O., Fryns, J.-P., Maldergem, L.V., Ziereisen, F., Yuksel, B., Gardiner, R.M., Chung, E. Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. *Nature Genetics* **21**, 142-144 (1999).

82. Hofmann Bowman, M., A., McNally, E.M. Genetic pathways of vascular calcification. *Trends in Cardiovascular Medicine* **22**, 93-98 (2012).

83. Lanzer, P., Boehm, M., Sorribas, V., Thiriet, M., Janzen, J., Zeller, T., St Hilaire, C., Shanahan, C. Medial vascular calcification revisited: review and perspectives. *European Heart Journal* **35**, 1515-1525 (2014).

84. Bertazzo, S., Gentleman, E., Cloyd, K.L., Chester, A.H., Yacoub, M.H., Stevens, M.M. Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. *Nature Materials* **12**, 576-583 (2013).

85. Schlieper, G., Aretz, A., Verberckmoes, S.C., Krueger, T., Behets, G.J., Ghadimi, R., Weirich, T.E., Rohrmann, D., Langer, S., Tordoir, J.H., Amann, K., Westenfield, R., Brandenburg, V.M., D'Haese, P.D., Mayer, J., Ketteler, M., McKee, M.D., Floege, J. Ultrastructural analysis of vascular calcifications in uremia. *Journal of American Society of Nephrology* **21**, 689-696 (2010).

86. Murshed, M., McKee, M.D. Molecular determinants of extracellular matrix mineralization in bone and blood vessels. *Current Opinion in Nephrology and Hypertension* **19**, 359-365 (2010).

87. Demer, L.L., Watson, K.E., Bostroem, K. Mechanism of calcification in atherosclerosis. *Trends in Cardiovascular Medicine* **4**, 45-49 (1994).

88. Hutcheson, J.D., Goettsh, C., Bertazzo, S., Maldonado, N., Ruiz, J.L., Goh, W., Yabusaki, K., Faits, T., Bouten, C., Franck, G., Quillard, T., Libby, P., Aikawa, M., Weinbaum, S., Aikawa, E. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. *Nature Materials* (2016).

89. Engler, A.J., Sen, S., Sweeney, H.L., Discher, D.E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **126**, 677-689 (2006).

90. Breitbach, M., Bostani, T., Roell, W., Xia, Y., Dewald, O., Nygren, J.M., Fries, J.W.U., Tiemann, K., Bohlen, H., Hescheler, J., Welz, A., Bloch, W., Jacobsen, S.E.W., Fleischmann, B.K. Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* **110**, 1362-1369 (2007).

91. Schoen, F.J., Levy, R.J. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. *Annals of Thoracic Surgery* **79**, 1072-1080 (2005).

92. Park, J.C., Siegel, R.J., Demer, L.L. Effect of calcification and formalin fixation on in vitro distensibility of human femoral arteries *American Heart Journal* **125**, 344-349 (1993).

93. Tam, H., Zhang, W., Feaver, K.R., Parchment, N., Sacks, M.S., Vyavahare, N. A novel crosslinking method for improved tear resistance and biocompatibility of tissue based biomaterials. *Biomaterials* **66**, 83-91 (2015).

94. Sundararaghavan, H.G., Monteiro, G.A., Lapin, N.A., Chabal, Y.J., Miksan, J.R., Shreiber, D.I. Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence. *J Biomed Mater Res Part A* **87**, 308-320 (2008).

95. Lim, H.G., Kim, S.H., Choi, S.Y., Kim, Y.J. Anticalcification effects of decellularization, solvent, and detoxification treatment for genipin and glutaraldehyde fixation of bovine pericardium. *European Journal of Cardiothoracic Surgery* **41**, 383-390 (2012).

96. Currey, J.D. The many adaptations of bone. *Journal of Biomechanics* **36**, 1487-95 (2003).

97. Levy, R.J., Schoen, F.J., Flowers, W.B., Staelin, S.T. Initiation of mineralization in bioprosthetic heart valves: Studies of alkaline phosphatase activity and its inhibition by A1Cl3 or FeC13 preincubations. *Journal of Biomedical Materials Research* **25**, 905-935 (1991).

98. Whyte, M.P., Greenberg, C.R., Salman, N.J., Bober, M.B., McAlister, W.H., Wenkert, D., Van Sickle, B.J., Simmons, J.H., Edgar, T.S., Bauer, M.L., Hamdan, M.A., Bishop, N., Lutz, R.E., McGinn, M., Craig, D., Moore, J.N., Taylor, J.W., Cleveland, R.H., Cranley, W.R., Lim, R., Thacher, T.D., Mayhew, J.E., Downs, M., Millán, J.L., Skrinar, A.M., Crine, P., Landy, H. Enzyme-replacement therapy in life-threatening hypophosphatasia. *N Engl J Med* **266**, 904-913 (2012).

99. Millán, J.L., Narisawa, S., Lemire, I., Loisel, T.P., Boileau, G., Leonard, P., Gramatikova, S., Terkeltaub, R., Pleshko Camacho, N., McKee, M.D., Crine, P., Whyte, M.P. Enzyme replacement therapy for murine hypophosphatasia. *J Bone Miner Res* **23**, 777-787 (2008).

100. Dash, M., Samal, S.K., Douglas, T.E.L., Schaubroeck, D., Leeuwenburgh, S.C., Van Der Voort, P., Declercq, H.A., Dubruel, P. Enzymatically biomineralized chitosan scaffolds for tissue-engineering applications. *Journal of Tissue Engineering and Regenerative Medicine* (2015).

101. Suárez-González, D., Barnhart, K., Migneco, F., Flanagan, C., Hollister, S.J., Murphy, W.L. . Controllable mineral coatings on PCL scaffolds as carriers for growth factor release. *Biomaterials* **33**, 713-721 (2012).

102. Liu, W., Yeh, Y.C., Lipner, J., Xie, J., Sung, H.W., Thomopoulos, S., et al. . Enhancing the stiffness of electrospun nanofiber scaffolds with a controlled surface coating and mineralization. *Langmuir* **27**, 9088-9093 (2011).

103. Gower, L.B. & Odum, D.J. Deposition of calcium carbonate films by a polymer-induced liquid-precursor (PILP) process. *J. Crystal Growth* **210**, 719-734 (2000).

104. Thula, T.T., Rodriguez, D.E., Lee, M.H., Pendi, L., Podschun, J., Gower, L.B. In vitro mineralization of dense collagen substrates: A biomimetic approach toward the development of bone-graft materials. *Acta Biomaterialia* **7**, 3158-3169 (2011).

105. Smith, L.A., Liu, X., Hu, J., Wang, P., Ma, P.X. Enhancing osteogenic differentiation of mouse embryonic stem cells by nanofibers. *Tissue Engineering Part A* **15**, 1855-1864 (2009).

106. Wei, G., Ma, P.X. Partially noanofibrous architecture of 3D tissue engineering scaffolds. *Biomaterials* **30**, 6426-6434 (2009).

107. Dalby, M.J., Gadegaard, N., Tare, R., Andar, A., Riehle, M.O., Herzyk, P., Wilkinson, C.D.W., Oreffo, R.O.C. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nature Materials* **6**, 997-1003 (2007).

108. Faia-Torres, A.B., Guimond-Lischer, S., Rottmar, M., Chamley, M., Goren, T., Maniura-Weber, K., Spencer, N.D., Reis, R.L., Textor, M., Neves, N.M. Differential regulation of osteogenic differentiation of stem cells on surface roughness gradients. *Biomaterials* **35**, 9023-9032 (2014).

109. Wirth, C., Comte, V., Lagneau, C., Exbrayat, P., Lissac, M., Jaffrezic-Renault, N., Ponsonnet, L. Nitinol surface roughness modulates in vitro cell response: a comparison between fibroblasts and osteoblasts. *Materials Science and Engineering C* **25**, 51-60 (2005).

110. Berner, A.K., Brouwers, O., Pringle, R., Klaassen, I., Colhoun, L., McVicar, C., Brockbank, S., Curry, J.W., Miyata, T., Brownlee, M., Schlingemann, R.O., Schalkwijk, C., Stitt, A.W. . Protection against methylglyoxal-derived AGEs by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. *Diabetologia* **55**, 845-854 (2012).

111. Nerlich, A.G., Zink, A., Szeimies, U., Hagedorn, H.G. Ancient Egyptian prosthesis of the big toe. *Lancet* **356**, 2176-79 (2000).

112. Nudelman, F., de With, G., Sommerdijk, N.A.J.M. Cryo-electron tomography: 3-dimensional imaging of soft matter. *Soft Matter* **7**, 17-24 (2011).

113. De Yoreo, J.J. & Sommerdijk, N.A.J.M. *Nature Reviews Materials* (2016).

114. Verch, A., Morrison, I.E.G., van de Locht, R. & Kröger, R. In situ electron microscopy studies of calcium carbonate precipitation from aqueous solution with and without organic additives. *J Struct Biol* **183**, 270-277 (2013).

115. Gentelman, E., Swain, R.J., Evans, N.D., Boonrungsiman, S., Jell, G., Ball, M.D., Shean, T.A.V., Oyen, M.L., Porter, A., Stevens, M.M. Comparative materials differences revealed in engineered bone as a function of cell-specific differentiation. *Nature Materials* **8**, 763-770 (2009).

116. Gordon, L.M., Joester, D. Nanoscale chemical tomography of buried organic–inorganic interfaces in the chiton tooth. *Nature* **469**, 194-197 (2011).

117. Roschger, P., Paschalis, E.P., Fratzl, P., Klaushofer, K. Bone mineralization density distribution in health and disease. *Bone* **42**, 456-466 (2008).

118. Achrai, B., Wagner, H.D. Micro-structure and mechanical properties of the turtle carapace as a biological composite shield. *Acta Biomaterialia* **9**, 5890-5902 (2013).

119. Wagermaier, W., Gupta, H.S., Gourrier, A., Burghammer, M., Roschger, P., Fratzl, P. Spiral twisting of fiber orientation inside bone lamellae. *Biointerphases* **1**, 1-5 (2006).

120. Stock, S.R., De Carlo, F., Almer, J.D. High energy X-ray scattering tomography applied to bone. *Journal of Structural Biology* **161**, 144-150 (2008).

121. Rinnerthaler, S., Roschger, P., Jakob, H. F., Nader, A., Klaushofer, K., Fratzl, P. Scanning small angle X-ray scattering analysis of human bone sections. *Calcif Tissue Int* **64**, 422-9 (1999).

122. Liebi, M., Georgiadis, M., Menzel, A., Schneider, P., Kohlbrecher, J., Bunk, O., Guizar-Sicairos, M. Nanostructure surveys of macroscopic specimens by small-angle scattering tensor tomography. *Nature* **527**, 349-352 (2015).

123. Li, C., Paris, O., Siegel, S., Roschger, P., Paschalis, E.P., Klaushofer, K., Fratzl,P. Strontium is incorporated into mineral crystals only in newly formed bone during strontium ranelate treatment. *Journal of Bone and Mineral Research* **25**, 968-975 (2009).

124. Camacho, N.P.R., S.; Paschalis, E.P.; Mendelsohn, R.; Boskey, A.L.; Fratzl, P. Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy. *Bone* **25**, 287-293 (1999).

125. Mueller, R. Hierarchical microimaging of bone structure and function. *Nature Reviews Rheumatology* **5**, 373-381 (2009).

126. Granke, M., Gourrier, A., Rupin, F., Raum, K., Peyrin, F., Burghammer, M., Saied, A., Laugier, P. Microfibril orientation dominates the microelastic properties of human bone tissue at the lamellar length scale. *PLoS ONE* **8**, e58046 (2013).

127. Georgiadis, M., Guizar-Sicairos, M., Zwahlen, A., Truessel, A.J., Bunk, O., Mueller, R., Schneider, P. 3D scanning SAXS: A novel method for the assessment of bone ultrastructure orientation. *Bone* **71**, 42-52 (2015).

128. Boskey, A.L. Bone composition: relationship to bone fragility and antiosteoporotic drug effects. *BoneKEy Reports* **2** (2013).

129. Kazanci, M. et al. Raman imaging of two orthogonal planes within cortical bone. *Bone* **41**, 456-461 (2007).

130. Weinkamer, R., Dunlop, J.W.C., Brechet, Y. & Fratzl, P. All but diamonds – Biological materials are not forever. *Acta Materialia* **61**, 880-889 (2013).

131. King, J.D., Bobechko, W.P. Osteogenesis Imperfecta. An orthopaedic description and surgical review. *Journal of Bone and Joint Surgery* **53-B**, 72-89 (1984).

132. Harmey, D., Hessle, L., Narisawa, S., Johnson, K.A., Terkeltaub, R., Millan, J.L. Concerted regulation of inorganic pyrophosphate and osteopontin by Akp2, Enpp1, and Ank. *American Journal of Pathology* **164**, 1199-1209 (2004).

References

1. Pearce, P. Structure in Nature Is a Strategy for Design (MIT Press, Boston, MA, USA, 1990).

2. Thompson, D.W. On Growth and Form (Cambridge University Press, Cambridge, 1942).

This monograph describes the natural phenomena of organisms' growth, development and morphology from a mathematical perspective and rationalizes the miracles of adaptation and diversity. It is a must-read for a bioengineer.

3. Campbell, A.K. Calcium as an intracellular regulator. Proceedings of the Nutrition Society 49, 51-56 (1990).

4. Penido, M.G.M.G., Alon, U.S. Phosphate homeostasis and its role in bone health. Pediatr Nephrol 27, 2039-2048 (2012).

5. Hunter, G.K., Kyle, C.L. & Goldberg, H.A. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochem J 300, 723-728 (1994).

6. Addison, W.N., Masica, D.L., Gray, J.J. & McKee, M.D. Phosphorylation-dependent inhibition of mineralization by osteopontin ASARM peptides is regulated by PHEX cleavage. J Bone Miner Res 25, 695-705 (2010).

7. Goldstein, D.A. Clinical Methods: The History, Physical, and Laboratory Examinations (Butterworths, Boston, MA, USA, 1990).

8. Magalhaes, M.C.F., Marques, P.A.A.P., Correira, R.N. Biomineralization. Medical Aspects of Solubility (ed. Koenigsberger, E., Koenigsberger, L.) (John Wiley and Sons Ltd., Chichester, UK, 2006).

9. Moreno, E.C., Gregory, T.M., Brown, W.E. Preparation and solubility of hydroxyapatite. Journal of Research of the National Bureau of Standards - A. Physics and Chemistry 72A, 773-782 (1968).

10. Elliott, J.C. Calcium phosphate biominerals. Rev. Geochem. Mineral. 48, 427-453 (2002).

11. Elliott, J.C. Hydroxyapatites and nonstoichiometric apatites. Studies in Inorganic Chemistry 18, 111-189 (1994).

12. Habraken, W.J.E.M., Tao, J., Brylka, L.J., Friedrich, H., Bertinetti, L., Schenk, A.S., Verch, A., Dmitrovic, V., Bomans, P.H.H., Frederik, P.M., Laven, J., van der Schoot, P., Aichmayer, B., de With, G., De Yoreo, J.J., Sommerdijk, N.A.J.M. Ion-association complexes unite classical and non-classical theories for the biomimetic nucleation of calcium phosphate. Nature Communications 4 (2013).

13. De Yoreo, J.J., Gilbert, P.U.P.A., Sommerdijk, N.A.J.M., Penn, R.L., Whitelam, S., Joester, D., Zhang, H., Rimer, J.D., Navrotsky, A., Banfield, J.F., Wallace, A.F., Michel, F.M., Meldrum, F.C., Coelfen, H., Dove, P.M. Crystallization by particle attachment in synthetic, biogenic, and geologic environments. Science 349, aaa6760 (2015).

14. Hodge, A.J. & Petruska, J.A. in Aspects of Protein Structure (ed. Ramachandran, G.N.) 289-300 (Academic Press, New York, 1963).

15. Saito, M., Marumo, K. Collagen cross-links as a determinant of bone quality: a possible explanation for bone fragility in aging, osteoporosis, and diabetes mellitus. Osteoporosis Int 21, 195-214 (2010).

16. Eyre, D.R., Dickson, I.R., Van Ness, K. Collagen cross-linking in human bone and articular cartilage. Biochem J 252, 495-500 (1988).

17. Fratzl, P. Collagen: structure and mechanics (Springer Science + Business Media, LLC, New York, USA, 2008).

18. Fratzl, P., Weinkamer, R. Nature's hierarchical materials. Progress in Materials Science 52, 1263-1334 (2007).

A comprehensive overview of natural materials, focusing on those where hierarchical organization of the structure is present.

19. Reznikov, N., Shahar, R., Weiner, S. Bone hierarchical structure in three dimensions. Acta Biomaterialia 10, 3815-2826 (2014).

20. Yamauchi, M., Chandler, G.S. & Katz, E.P. in Chemistry and Biology of Mineralized Tissues. (eds. Slavkin, H. & P., P.) 39-46 (Elsever Science Publishers, 1992).

21. Fleisch, H., Russell, R.G.G., Straumann, F. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature 5065, 901-903 (1966).

22. Omelon, S. et al. Control of vertebrate skeletal mineralization by polyphosphates. PLoS One 4, e5634 (2009).

23. Omelon, S., Ariganello, M., Bonucci, E., Grynpas, M., Nanci, A. A review of phosphate mineral nucleation in biology and geobiology. Calcified Tissue International 93, 382-396 (2013).

24. Jahnen-Dechent, W., Schaefer, C., Ketteler, M., McKee, M.D. Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. Journal of Molecular Medicine 86, 379-389 (2008).

25. Jahnen-Dechent, W., Heiss, A., Schaefer, C., Ketteler, M. Fetuin A regulation of calcified matrix metabolism. Circulation Research 108, 1494-1509 (2011).

26. Millan, J.L. Alkaline phosphatases. Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. Purinergic Signalling 2, 335-341 (2006).

27. George, A., Veis, A. Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition. Chem Rev 108, 4670-4693 (2008).

28. Nudelman, F. et al. The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. Nature Materials 9, 1004-1009 (2010).

29. Wang, Y., Azais, T., Robin, M., Vallee, A., Catania, C., Legriel, P., Pehau-Arnaudet, G., Babonneau, F., Giraud-Guille, M.-M., Nassif, N. The predominant role of collagen in the nucleation, growth, structure and orientation of bone apatite. Nature Materials 11, 724-733 (2012).

Interaction of organic, inorganic phases and water are discussed with an emphasis on self-assembly processes and the role of water in biomineralization.

30. McKee, M.D., Nanci, A. Osteopontin: an interfacial extracellular matrix protein in mineralized tissues. Connect Tissue Res 35, 197-205 (1996).

31. McKee, M.D., Nanci, A. Osteopontin at mineralized tissue interfaces in bone, teeth and osseointegrated implants: ultrastructural distribution and implications for mineralized tissue formation, turnover and repair. Microscopy Reasearch and Technique 33, 141-164 (1996).

32. McKee, M.D., Zalzal, S., Nanci, A. Extracellular matrix in tooth cementum and mantle dentin: localization of osteopontin and other noncollagenous proteins, plasma proteins, and glycoconjugates by electron microscopy. The Anatomical Record 245, 293-312 (1996).

33. Kavukcuoglu, N.B., Denhardt, D.T., Guzelsu, N. & Mann, A.B. Osteopontin deficiency and aging on nanomechanics of mouse bone. J Biomed Mater Res A 83, 136-44 (2007).

34. Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B., McKee, M.D. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcified Tissue International 71, 145-154 (2002).

35. Luo, G., Ducy, P., McKee, M.D., Pinero, G.J., Loyer, E., Behringer, R.R., Karsenty, G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. Nature 386, 78-81 (1997).

36. Schinke, T., McKee, M.D., Karsenty, G. Extracellular matrix calcification: where is the action? Nature Genetics 21, 150-151 (1999).

37. Gorski, J.P. Is all bone the same? Distinctive distributions and properties of non-collagenous matrix proteins in lamellar vs. woven bone imply the existence of different underlying osteogenic mechanisms. Critical Reviews in Oral Biology and Medicine 9, 201-23 (1998).

38. Boskey, A.L. Biomineralization:conflicts, challenges and opportunities. J. Cell. Biochem. Suppl. 30-31, 83-91 (1998).

39. Veis, A. in Biomineralization. Reviews in Mineralogy and Geochemistry (eds. Dove, P.M., De Yoreo, J.J. & Weiner, S.) 249-290 (Mineralogical Society of America, Washington DC, 2003).

40. Barros, N.M.T., Hoac, B., Neves, R.L., Addison, W.N., Assis, D.M., Murshed, M., Carmona, A.K., McKee, M.D. Proteolytic processing of osteopontin by PHEX and accumulation of osteopontin fragments in Hyp mouse bone, the murine model of X-linked hypophosphatemia. Journal of Bone and Mineral Research 28, 688-699 (2013).

41. Posner, A.S., Betts, F., Blumenthal, N.C. Properties of nucleating systems. Metabolic Bone Disease 1, 179-183 (1978).

42. Betts, F., Blumenthal, N.C., Posner, A.S. Bone mineralization. Journal of Crystal Growth 53, 63-73 (1981).

43. Mahamid, J., Sharir, A., Gur, D., Zelzer, E., Addadi, L., Weiner, S. Bone mineralization proceeds through intracellular calcium phosphate loaded vesicles: a cryo-electron microscopy study. J Struct Biol 174, 527-535 (2011).

44. Politi, Y., Arad, T., Klein, E., Weiner, S. & Addadi, L. Sea urchin spine calcite forms via a transient amorphous calcium carbonate phase. Science 306, 1161-4 (2004).

45. Mahamid, J., Sharir, A., Addadi, L., Weiner, S. Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: indications for an amorphous precursor phase. PNAS 105, 12748-12753 (2008).

This state-of-the-art cryo-electron microscopy study of bone formation and maturation in situ clearly illustrates the dynamics of biomineralization via the disordered mineral precursor stage.

46. Boonrungsiman, S., Gentleman, E., Carzaniga, R., Evans, N.D., McComb, D.W., Porter, A.E., Stevens, M.M. The role of intracellular calcium phosphate in osteoblast-mediated bone apatite formation. PNAS 109, 14170-14175 (2012).

47. Akiva, A., Malkinson, G., Masic, A., Kerschnitzki, M., Bennet, M., Fratzl, P., Addadi, L., Weiner, S., Yaniv, K. On the pathway of mineral deposition in larval zebrafish caudal fin bone. Bone 75, 192-200 (2015).

48. Kerschnitzki, M., Akiva, A., Ben Shoham, A., Koifman, N., Shimoni, E., Rechav, K., Arraf, A.A., Schultheiss, T.M., Talmon, Y., Zelzer, E., Weiner, S., Addadi, L/. Transport of membrane-bound mineral particles in blood vessels during chicken embryonic bone development. Bone 83, 65-72 (2016).

49. Currey, J.D. Bones: Structure and Mechanics (Princeton University press, Oxford, 2002).

50. Wagermaier, W., Klaushofer, K., Fratzl, P. Fragility of bone material controlled by internal interfaces. Calcified Tissue International 97, 201-212 (2015).

This paper overviews the role of interfaces at multiple size scales that provide appropriate bone toughness and provides a paradigm shift from the common-place notion of "strength" towards an accurate vision of the bone quality determinants.

51. Martin, R.B., Burr, D.B. & Sharkey, N.A. Skeletal Tissue Mechanics (Springer-Verlag, New York, 1998).

52. Helfrich, M.H. Osteoclast diseases. Microscopy Research and Technique 61, 514-32 (2003).

53. Golub, E.E. Role of matrix vesicles in biomineralization. Biochimica et Biophysica Acta 1790, 1592-1598 (2009).

54. Landis, W.J., Hodgens, K.J., Arena, J., Song, M.J. & McEwen, B.F. Structural relations between collagen and mineral in bone as determined by high voltage electron microscopic tomography. Microsc. Res. Tech. 33, 192-202 (1996).

55. Weiner, S. & Price, P. Disaggregation of bone into crystals. Calcif Tissue Int. 39, 365-375 (1986).

56. Moradian-Oldak, J., Weiner, S., Addadi, L., Landis, W.J. & Traub, W. Electron imaging and diffraction study of individual crystals of bone, mineralized tendon and synthetic carbonate apatite. Connect Tissue Res 25, 219-28 (1991).

57. Wang, Y., Von Euw, S., Fernandes, F.M., Cassaignon, S., Selmane, M., Laurent, G., Pehau-Arnaudet, G., Coelho, C., Bonhomme-Coury, L., Giraud-Guille, M.-M., Babonneau, F., Azais, T., Nassif, N. Water-mediated structuring of bone apatite. Nature Materials 12, 1144-1153 (2013).

58. Ennos, R. Solid biomechanics (Princeton University Press, Woodstock, England, 2012).

59. Wainwright, S.A., Biggs, W.D., Currey, J.D. & Gosline, J.M. Mechanical Design in Organisms (Princeton University Press, Princeton, 1976).

An insightful monograph recommended for junior scientists working in the fields of (bio)materials and (bio)engineering. Nature's answers to engineering problems are presented in the context of basic physics and converted into a source of inspiration for materials design.

60. Gordon, J.E. Structures: or why things don't fall down (Da Capo Press, Reading, England, 2003).

This witty textbook explains memorable real life examples of engineering issues in an entertaining manner yet with adamant logic; recommended for life scientists tackling the fields of engineering and materials science.

61. Nikitovic, D., Aggelidakis, J., Young, M.F., Iozzo, R.V., Karamanos, N.K., Tzanakakis, G.N. The biology of small leucine-rich proteoglycans in bone pathophysiology. Journal of Biological Chemistry 287, 33926-33933 (2012).

62. Allen, M.R., Newman, C.L., Chen, N., Granke, M., Nyman, J.S., Moe, S.M. Changes in skeletal collagen cross-links and matrix hydration in high- and low-turnover chronic kidney disease. Osteoporosis Int 26, 977-985 (2015).

63. Bertinetti, L. et al. Osmotically driven tensile stress in collagen-based mineralized tissues. J Mech Behav Biomed Mater 52, 14-21 (2015).

64. Masic, A., Berrtinetti, L., Schuetz, R., Chang, S.-W., Metzger, T.H., Buehler, M.J., Fratzl, P. Osmotic pressure induces tensile forces in tendon collagen. Nature Communications, 6:5942 (2015).

65. Maroudas, A. Balance between swelling pressure and collagen tension in normal and degenerate cartilage. Nature 260, 808-809 (1976).

66. Timmins, P.A. & Wall, J.C. Bone water. Calcified Tissue Research 23, 1-5 (1977).

67. Forien, J.B. et al. Compressive residual strains in mineral nanoparticles as a possible origin of enhanced crack resistance in human tooth dentin. Nano Letters 15, 3729-3734 (2015).

68. Kaartinen, M.T., El-Maadawy, S., Rasanen, N.H., McKee, M.D. Tissue transglutaminase and its substrates in bone. Journal of Bone and Mineral Research 17, 2161-2173 (2002).

69. Gupta, H.S., Seto, J., Wagermaier, W., Zaslansky, P., Boesecke, P., Fratzl, P. Cooperative deformation of mineral and collagen in bone at the nanoscale. PNAS 103, 17741-17746 (2006).

70. Hoo, R.P., Fratzl, P., Daniels, J.E., Dunlop, J.W.C., Honkimaeki, V., Hoffmann, M. Cooperation of length scales and orientations in the deformation of bovine bone. Acta Biomaterialia 7, 2943-2951 (2011).

71. Reznikov, N., Almany-Magal, R., Shahar, R., Weiner, S. Three-dimensional imaging of collagen fibril organization in rat circumferential lamellar bone using a dual beam electron microscope reveals ordered and disordered sub-lamellar structures. Bone 52, 676-683 (2013).

72. Reznikov, N., Shahar, R., Weiner, S. Three-dimensional structure of human lamellar bone: the presence of two different materials and new insights into the hierarchical organization. Bone 59 (2014).

73. Utku, F.S., Klein, E., Saybasili, H., Yucesoy, C.A., Weiner, S. Probing the role of water in lamellar bone by dehydration in the environmental scanning electron microscope. J Struct Biol 162, 361-367 (2008).

74. Johnson, R.C., Leopold, J.A., Loscalzo, J. Vascular calcification. Pathobiological mechanisms and clinical implications. Circulation Research 90, 1044-1059 (2006).

75. Lee, D. Vascular calcification: Inducers and inhibitors. Materials Science and Engineering B 176, 1133-1141 (2011).

76. Marulanda, J., Alqarni, S., Murshed, M. Mechanisms of vascular calcification and associated diseases. Current Pharmaceutical Design 20, 1-10 (2014).

77. Ruiz, J.L., Hutcheson, J.D., Aikawa, E. Cardiovascular calcification: current controversies and novel concepts. Cardiovascular Pathology 24, 207-212 (2015).

78. Clarke, M.C.H., Littlewood, T.D., Figg, N., Maguire, J.J., Davenport, A.P., Goddard, M., Bennett, M.R. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. Circulation Research 102, 1529-1538 (2008).

79. Liaw, L., Lindner, V., Schwartz, S.M., Chambers, A.F., Giachelli, C.M. Osteopontin and beta 3 integrin are coordinately expressed in regenerating endothelium in vivo and stimulate Arg-Gly-Asp-dependent endothelial migration in vitro. Circulation Research 77, 665-672 (1995).

80. Singleton, E.B., Merten, D.F. An unusual syndrome of widened medullary cavities of the metacarpals and phalanges, aortic calcification and abnormal dentition. Pediatric Radiology 1, 2-7 (1973).

81. Munroe, P.B., Olgunturk, R.O., Fryns, J.-P., Maldergem, L.V., Ziereisen, F., Yuksel, B., Gardiner, R.M., Chung, E. Mutations in the gene encoding the human matrix Gla protein cause Keutel syndrome. Nature Genetics 21, 142-144 (1999).

82. Hofmann Bowman, M., A., McNally, E.M. Genetic pathways of vascular calcification. Trends in Cardiovascular Medicine 22, 93-98 (2012).

83. Lanzer, P., Boehm, M., Sorribas, V., Thiriet, M., Janzen, J., Zeller, T., St Hilaire, C., Shanahan, C. Medial vascular calcification revisited: review and perspectives. European Heart Journal 35, 1515-1525 (2014).

84. Bertazzo, S., Gentleman, E., Cloyd, K.L., Chester, A.H., Yacoub, M.H., Stevens, M.M. Nano-analytical electron microscopy reveals fundamental insights into human cardiovascular tissue calcification. Nature Materials 12, 576-583 (2013).

This work is an example how traditional materials-oriented analytical electron microscopy techniques aid in resolving long-standing disputes of structural biology.

85. Schlieper, G., Aretz, A., Verberckmoes, S.C., Krueger, T., Behets, G.J., Ghadimi, R., Weirich, T.E., Rohrmann, D., Langer, S., Tordoir, J.H., Amann, K., Westenfield, R., Brandenburg, V.M., D'Haese, P.D., Mayer, J., Ketteler, M., McKee, M.D., Floege, J. Ultrastructural analysis of vascular calcifications in uremia. Journal of American Society of Nephrology 21, 689-696 (2010).

86. Murshed, M., McKee, M.D. Molecular determinants of extracellular matrix mineralization in bone and blood vessels. Current Opinion in Nephrology and Hypertension 19, 359-365 (2010).

This concise work underscores the common features between physiological and pathological mineralization and highlights the role of non-collagenous proteins in both

87. Demer, L.L., Watson, K.E., Bostroem, K. Mechanism of calcification in atherosclerosis. Trends in Cardiovascular Medicine 4, 45-49 (1994).

88. Hutcheson, J.D., Goettsh, C., Bertazzo, S., Maldonado, N., Ruiz, J.L., Goh, W., Yabusaki, K., Faits, T., Bouten, C., Franck, G., Quillard, T., Libby, P., Aikawa, M., Weinbaum, S., Aikawa, E. Genesis and growth of extracellular-vesicle-derived microcalcification in atherosclerotic plaques. Nature Materials (2016).

89. Engler, A.J., Sen, S., Sweeney, H.L., Discher, D.E. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell 126, 677-689 (2006).

90. Breitbach, M., Bostani, T., Roell, W., Xia, Y., Dewald, O., Nygren, J.M., Fries, J.W.U., Tiemann, K., Bohlen, H., Hescheler, J., Welz, A., Bloch, W., Jacobsen, S.E.W., Fleischmann, B.K. Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 110, 1362-1369 (2007).

91. Schoen, F.J., Levy, R.J. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. Annals of Thoracic Surgery 79, 1072-1080 (2005).

92. Park, J.C., Siegel, R.J., Demer, L.L. Effect of calcification and formalin fixation on in vitro distensibility of human femoral arteries American Heart Journal 125, 344-349 (1993).

93. Tam, H., Zhang, W., Feaver, K.R., Parchment, N., Sacks, M.S., Vyavahare, N. A novel crosslinking method for improved tear resistance and biocompatibility of tissue based biomaterials. Biomaterials 66, 83-91 (2015).

94. Sundararaghavan, H.G., Monteiro, G.A., Lapin, N.A., Chabal, Y.J., Miksan, J.R., Shreiber, D.I. Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence. J Biomed Mater Res Part A 87, 308-320 (2008).

95. Lim, H.G., Kim, S.H., Choi, S.Y., Kim, Y.J. Anticalcification effects of decellularization, solvent, and detoxification treatment for genipin and glutaraldehyde fixation of bovine pericardium. European Journal of Cardiothoracic Surgery 41, 383-390 (2012).

96. Currey, J.D. The many adaptations of bone. Journal of Biomechanics 36, 1487-95 (2003).

97. Levy, R.J., Schoen, F.J., Flowers, W.B., Staelin, S.T. Initiation of mineralization in bioprosthetic heart valves: Studies of alkaline phosphatase activity and its inhibition by A1Cl3 or FeC13 preincubations. Journal of Biomedical Materials Research 25, 905-935 (1991).

98. Whyte, M.P., Greenberg, C.R., Salman, N.J., Bober, M.B., McAlister, W.H., Wenkert, D., Van Sickle, B.J., Simmons, J.H., Edgar, T.S., Bauer, M.L., Hamdan, M.A., Bishop, N., Lutz, R.E., McGinn, M., Craig, D., Moore, J.N., Taylor, J.W., Cleveland, R.H., Cranley, W.R., Lim, R., Thacher, T.D., Mayhew, J.E., Downs, M., Millán, J.L., Skrinar, A.M., Crine, P., Landy, H. Enzyme-replacement therapy in life-threatening hypophosphatasia. N Engl J Med 266, 904-913 (2012).

This work is a striking illustration of a translational application of basic biomineralization research.

99. Millán, J.L., Narisawa, S., Lemire, I., Loisel, T.P., Boileau, G., Leonard, P., Gramatikova, S., Terkeltaub, R., Pleshko Camacho, N., McKee, M.D., Crine, P., Whyte, M.P. Enzyme replacement therapy for murine hypophosphatasia. J Bone Miner Res 23, 777-787 (2008).

100. Dash, M., Samal, S.K., Douglas, T.E.L., Schaubroeck, D., Leeuwenburgh, S.C., Van Der Voort, P., Declercq, H.A., Dubruel, P. Enzymatically biomineralized chitosan scaffolds for tissue-engineering applications. Journal of Tissue Engineering and Regenerative Medicine (2015).

101. Suárez-González, D., Barnhart, K., Migneco, F., Flanagan, C., Hollister, S.J., Murphy, W.L. . Controllable mineral coatings on PCL scaffolds as carriers for growth factor release. Biomaterials 33, 713-721 (2012).

102. Liu, W., Yeh, Y.C., Lipner, J., Xie, J., Sung, H.W., Thomopoulos, S., et al. . Enhancing the stiffness of electrospun nanofiber scaffolds with a controlled surface coating and mineralization. Langmuir 27, 9088-9093 (2011).

103. Gower, L.B. & Odum, D.J. Deposition of calcium carbonate films by a polymer-induced liquid-precursor (PILP) process. J. Crystal Growth 210, 719-734 (2000).

104. Thula, T.T., Rodriguez, D.E., Lee, M.H., Pendi, L., Podschun, J., Gower, L.B. In vitro mineralization of dense collagen substrates: A biomimetic approach toward the development of bone-graft materials. Acta Biomaterialia 7, 3158-3169 (2011).

105. Smith, L.A., Liu, X., Hu, J., Wang, P., Ma, P.X. Enhancing osteogenic differentiation of mouse embryonic stem cells by nanofibers. Tissue Engineering Part A 15, 1855-1864 (2009).

106. Wei, G., Ma, P.X. Partially noanofibrous architecture of 3D tissue engineering scaffolds. Biomaterials 30, 6426-6434 (2009).

107. Dalby, M.J., Gadegaard, N., Tare, R., Andar, A., Riehle, M.O., Herzyk, P., Wilkinson, C.D.W., Oreffo, R.O.C. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. Nature Materials 6, 997-1003 (2007).

108. Faia-Torres, A.B., Guimond-Lischer, S., Rottmar, M., Chamley, M., Goren, T., Maniura-Weber, K., Spencer, N.D., Reis, R.L., Textor, M., Neves, N.M. Differential regulation of osteogenic differentiation of stem cells on surface roughness gradients. Biomaterials 35, 9023-9032 (2014).

109. Wirth, C., Comte, V., Lagneau, C., Exbrayat, P., Lissac, M., Jaffrezic-Renault, N., Ponsonnet, L. Nitinol surface roughness modulates in vitro cell response: a comparison between fibroblasts and osteoblasts. Materials Science and Engineering C 25, 51-60 (2005).

110. Berner, A.K., Brouwers, O., Pringle, R., Klaassen, I., Colhoun, L., McVicar, C., Brockbank, S., Curry, J.W., Miyata, T., Brownlee, M., Schlingemann, R.O., Schalkwijk, C., Stitt, A.W. . Protection against methylglyoxal-derived AGEs by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. Diabetologia 55, 845-854 (2012).

111. Nerlich, A.G., Zink, A., Szeimies, U., Hagedorn, H.G. Ancient Egyptian prosthesis of the big toe. Lancet 356, 2176-79 (2000).

112. Nudelman, F., de With, G., Sommerdijk, N.A.J.M. Cryo-electron tomography: 3-dimensional imaging of soft matter. Soft Matter 7, 17-24 (2011).

113. De Yoreo, J.J. & Sommerdijk, N.A.J.M. Nature Reviews Materials (2016).

114. Verch, A., Morrison, I.E.G., van de Locht, R. & Kröger, R. In situ electron microscopy studies of calcium carbonate precipitation from aqueous solution with and without organic additives. J Struct Biol 183, 270-277 (2013).

115. Gentelman, E., Swain, R.J., Evans, N.D., Boonrungsiman, S., Jell, G., Ball, M.D., Shean, T.A.V., Oyen, M.L., Porter, A., Stevens, M.M. Comparative materials differences revealed in engineered bone as a function of cell-specific differentiation. Nature Materials 8, 763-770 (2009).

116. Gordon, L.M., Joester, D. Nanoscale chemical tomography of buried organic–inorganic interfaces in the chiton tooth. Nature 469, 194-197 (2011).

117. Roschger, P., Paschalis, E.P., Fratzl, P., Klaushofer, K. Bone mineralization density distribution in health and disease. Bone 42, 456-466 (2008).

118. Achrai, B., Wagner, H.D. Micro-structure and mechanical properties of the turtle carapace as a biological composite shield. Acta Biomaterialia 9, 5890-5902 (2013).

119. Wagermaier, W., Gupta, H.S., Gourrier, A., Burghammer, M., Roschger, P., Fratzl, P. Spiral twisting of fiber orientation inside bone lamellae. Biointerphases 1, 1-5 (2006).

120. Stock, S.R., De Carlo, F., Almer, J.D. High energy X-ray scattering tomography applied to bone. Journal of Structural Biology 161, 144-150 (2008).

121. Rinnerthaler, S., Roschger, P., Jakob, H. F., Nader, A., Klaushofer, K., Fratzl, P. Scanning small angle X-ray scattering analysis of human bone sections. Calcif Tissue Int 64, 422-9 (1999).

122. Liebi, M., Georgiadis, M., Menzel, A., Schneider, P., Kohlbrecher, J., Bunk, O., Guizar-Sicairos, M. Nanostructure surveys of macroscopic specimens by small-angle scattering tensor tomography. Nature 527, 349-352 (2015).

123. Li, C., Paris, O., Siegel, S., Roschger, P., Paschalis, E.P., Klaushofer, K., Fratzl,P. Strontium is incorporated into mineral crystals only in newly formed bone during strontium ranelate treatment. Journal of Bone and Mineral Research 25, 968-975 (2009).

124. Camacho, N.P.R., S.; Paschalis, E.P.; Mendelsohn, R.; Boskey, A.L.; Fratzl, P. Complementary information on bone ultrastructure from scanning small angle X-ray scattering and Fourier-transform infrared microspectroscopy. Bone 25, 287-293 (1999).

125. Mueller, R. Hierarchical microimaging of bone structure and function. Nature Reviews Rheumatology 5, 373-381 (2009).

126. Granke, M., Gourrier, A., Rupin, F., Raum, K., Peyrin, F., Burghammer, M., Saied, A., Laugier, P. Microfibril orientation dominates the microelastic properties of human bone tissue at the lamellar length scale. PLoS ONE 8, e58046 (2013).

127. Georgiadis, M., Guizar-Sicairos, M., Zwahlen, A., Truessel, A.J., Bunk, O., Mueller, R., Schneider, P. 3D scanning SAXS: A novel method for the assessment of bone ultrastructure orientation. Bone 71, 42-52 (2015).

128. Boskey, A.L. Bone composition: relationship to bone fragility and antiosteoporotic drug effects. BoneKEy Reports 2 (2013).

129. Kazanci, M. et al. Raman imaging of two orthogonal planes within cortical bone. Bone 41, 456-461 (2007).

130. Weinkamer, R., Dunlop, J.W.C., Brechet, Y. & Fratzl, P. All but diamonds – Biological materials are not forever. Acta Materialia 61, 880-889 (2013).

Perfection is not necessarily a virtue. A broad understanding of biological materials structure underscores how "defects" can be taken advantage of.

131. King, J.D., Bobechko, W.P. Osteogenesis Imperfecta. An orthopaedic description and surgical review. Journal of Bone and Joint Surgery 53-B, 72-89 (1984).

132. Harmey, D., Hessle, L., Narisawa, S., Johnson, K.A., Terkeltaub, R., Millan, J.L. Concerted regulation of inorganic pyrophosphate and osteopontin by Akp2, Enpp1, and Ank. American Journal of Pathology 164, 1199-1209 (2004).

Box 1 Cardiovascular anatomy and physiology

The innermost surface of an artery is covered by endothelium of one-cell thickness, which is antithrombogenic. Endothelium is supported by a basement membrane – a thin meshwork layer of collagens type III, IV and V. This tissue complex is called the *tunica intima*. The middle layer, or *tunica media,* includes the internal elastic lamina (a sub-layer of anastomosing elastin fibrils found immediately under the basement membrane), followed by multiple layers of vascular smooth muscle

cells alternating with concentric and undulating elastic laminae residing in an extracellular matrix of crimpled collagen type I fibrils and proteoglycans, collectively bounded at its outermost region by the external elastic lamina. The third major layer, or *tunica adventitia*, is the outer connective tissue sleeve of the artery consisting predominantly of abundant collagen type I fibrils, fibroblasts, neural endings and capillaries. The role of *tunica adventitia* is auxiliary, whereas the *tunica media* bears most of the mechanical burden. During systole (contraction of the ventricles), the arterial wall expands compliantly to prevent a catastrophic build-up of hydraulic pressure; however, the extent of expansion is defined by the concentric arrays of non-stretchable collagen fibrils (high tensile strength of arterial collagen prevents local bulging and lowers the risk of developing an aneurysm). During diastole (relaxation of the ventricles), previously extended elastin fibrils in the elastic laminae of the media recoil elastically, and the lumen diameter then again becomes smaller, until the next systole initiates a new cycle and a new pulsatile wave. Thus, repetitive limited expansion juxtaposed by the steady recoil of the arterial media together maintain blood pressure within the physiological range. The layer of smooth muscle cells regulates the limits of the arterial diameter between the systolic and diastolic phases, depending on the volume of circulating blood, thermoregulation and neural input.

The heart has two atrio-ventricular valves that preclude blood from re-entering the veins during systole, and aortic and pulmonary valves that prevent retrograde blood flow from the aorta and the pulmonary trunk back into the heart during diastole. Aortic, pulmonary and tricuspid valves are comprised of three leaflets, and the mitral valve has two leaflets. The central functional valve parts (cusps) of the four valves are attached to stiff fibrous outer rings – the 'skeleton' of the heart – and are lined by endocardium (a one-cell-thick layer, similar to endothelium). The heart completes up to 3 billion beats over its lifetime, with the passage of 3-5 litres of blood per minute imparting enormous shear stress on the valve surfaces. Of all the heart valves, the highest tensile stress (ca. 500 kPa) is exerted on the non-flow surface of the aortic valve in diastole. The high biomechanical requirements of the aortic valve are reflected in its structure. The non-flow aspect of the aortic valve is called the *fibrosa* and comprises circumferentially aligned collagen fibrils – its flow surface is called the ventricularis, which contains a less-ordered array of mixed collagen and elastin fibrils. Between the *fibrosa* and the *ventricularis* lies the spongiosa, a layer of extracellular matrix that is rich in proteoglycans. The thickness of all three layers does not normally exceed 1 mm in humans; the leaflets are avascular and receive the nutrients by diffusion from the nearby passing blood.

Figure Captions (refer to published version for Figures)

Figure 1. Physiological regulation of mineralization. Regions of biomineralization promotion are depicted in blue and regions of biomineralization inhibition are depicted in red. a, Elastic arterial walls in vascular networks are examples of the inhibition of mineralization of the extracellular matrix. b, Bone tissue, composed of haversian systems and osteons, is an example of the promotion of extracellular matrix mineralization. Both soft and hard tissues are perfused by blood containing bound and sequestrated inorganic ions; binding and sequestration of calcium (green) and phosphate (grey) ions prevents spontaneous formation of minerals[25]. In cardiovascular tissues, mineralization is further prevented by the resident cells, vascular smooth muscle cells which secrete local inhibitors such as matrix Gla protein[35]. In bone tissue, the inhibitory effect of calcium and phosphorus sequestration is abolished by the enzymatic activity of the resident cells, osteoblasts, and extracellular matrix mineralization is facilitated locally.

Figure 2. Cyclic loading in the cardiovascular system and the effects of mineralization. a) A schematic representation of the arterial wall during systole and diastole. In the arterial wall, the systolic blood pressure build up is counteracted by the hoop strain of the stretched collagen and elastin fibres. During diastole, decreasing blood pressure is followed by recoil of elastic fibres and passive crimpling of collagen fibres. b) A schematic representation of the

cardiac valves during systole and diastole. During systole, the collagen fibres of the non-flow surface crimple, and the elastin fibres of the flow surface stretch. During diastole, the collagen fibres of the non-flow surface stretch and the elastin fibres of the flow surface relax. c) Clinical examples in which the normal mechanical response of the tissues is impaired by the detrimental effects of pathological mineralization.

Figure 3. Examples of correlative combinations of materials-based analytical tools for multi-scale interrogation of bone. The arrows indicate how a higher-resolution image can be contextualized within a broader-scale image in order to keep track of the organizational level within a complex hierarchical material such as bone.

Figure 4. Unanswered questions related to the pathological mineralization of cardiovascular tissues and the physiological mineralization of bone. a, The arterial wall. Constituents that are associated with pathological mineralization are depicted in blue— these include globular calcium phosphate particles, diffuse mineralization (collagen and elastin) of the arterial wall, and pro-osteogenic cells. b, The formation of bone. Constituents that pose unanswered questions include mineral-containing vesicles in the peripheral bloodstream, osteoblasts and osteocytes, and the canalicular network.

Tables

Table 1. An overview of clinical conditions associated with perturbed skeletal tissue mineralization in comparison with physiological mineralization.

	Adequate	Perturbed	Deficient			Excessive	
Clinical condition	**Physiological mineralization of bone and teeth**	Osateoporosis	Vitamin-D deficient rickets	Osteogenesis imperfecta	X-linked hypophosphataemia; hypophosphatasia	Osteopetrosis	Ankylosis
Calcium-phosphorus ionic product	**-Metastable -Hyper-saturation with respect to hydroxyapatite**		Low		Low serum phosphate		
Collagen framework	**-Organized -Crosslinked**	-Accumulates AGE		Impaired crosslinking			
Systemic inhibitors	**-Active**						Low pyrophosphate
Local inhibitors	**-Active - Form local gradients**	-Loss of gradients -Loss of interfaces					Impaired
Local promoters	**-Abundant**				Impaired	Elevated	Elevated
Patho-physiological background	**N/A**	Perturbed remodeling	Metabolic	Genetic	Genetic	Genetic	Genetic or traumatic
Mechanical implications in tissues	**-Optimal stiffness -High toughness**	-High stiffness -Low toughness -Low total bone mass	Low stiffness	Low toughness	Low stiffness	Low toughness	Inappropriate stiffness

References	**49,51**	15, 50	4	131	40, 98	52	132

Footnotes: empty field means consistent with the normal state; AGE stands for advanced glycation endproducts

Table 2 An overview of clinical conditions associated with cardiovascular mineralization, which is always excessive.

Clinical condition	Medial or valvular calcification	Atherosclerotic calcification	Calcification of valvular and vascular prostheses
Calcium-phosphorus ionic product	Elevated serum phosphate		
Collagen framework	May accummulate AGE (crosslinking)	May accummulate AGE (crosslinking)	Chemically crosslinked
Systemic inhibitors			
Local inhibitors	Depleted	Depleted	Inactive
Local promoters	Can be elevated	Can be elevated	Can be elevated
Pathophysiological background	Metabolic or genetic	-Metabolic -Local tissue inflammation and necrosis	
Mechanical implications in tissues	-High siffness -Low resilience -Low relaxation rate	-High siffness -Low resilience -Low relaxation rate	-High siffness -Low resilience -Low relaxation rate
References	75, 76, 87, 82		91

Footnotes: empty field means consistent with the normal state; AGE stands for advanced glycation endproduct

Table 3. The role of natural and artificial crosslinks in materials properties

	Crosslinking			
	Natural			**Artificial**
	Physiological		**Pathological**	**Chemical fixation**
Agent	Lysyl Oxidase (LO)	Transglutaminase II	Advanced Glycation Endproducts (AGE)	Aldehydes
Substrate	Collagen, elastin	Noncollagenous proteins, collagen	Proteins, lipids, nucleic acids	Proteins, lipids, nucleic acids
Location	Tissue-specific		Non-tissue-specific	
Biomechanical effect	Increased tensile modulus		Abnormally high stiffness, incomplete recoil	

	Increased toughness		Decreased toughness	
Chemical specificity	(Hydroxy)lysyl residues in specific positions. Requires supramolecular assembly	γ-carboxamide residues and primary amines (*e.g.* lysyl) of osteocalcin, OPN, BSP, fetuin A, *etc.*	Non-substrate-specific; Binds to terminal amino groups and forms cyclic crosslinks	Non-substrate-specific; Binds to terminal amino groups
Notes	Rapid accumulation during tissue development. Immature divalent crosslinks rearrange to form mature trivalent crosslinks. Hereditary defect: Ehlers-Danlos syndrome [16] [17]	Form intramolecular bonds to stabilize the substrate and intermolecular bonds to form large aggregates/ polymers. Contributes to sacrificial bonds in the extracellular matrix [68]	Analogous to the Maillard reaction. Gradual accumulation with ageing. Accelerated accumulation in glycaemia. Associated with slow tissue turnover. Affects all tissues rich in extracellular matrix [15] [62]	Traditionally used for tissue preservation, stabilization and sterilization. Decreases antigen recognition. Non-fixed grafts are resorbed by the host. Glutaraldehyde-fixed grafts mineralize [91] [92]

Table 4. Materials-based analytical tools in the order of increasing resolution. The asterisks indicate the methods applicable to living models/cultures.

Analytical technique	**Output**	**2D**	**3D**
Atom probe tomography	Elemental composition		[116]
Electron energy loss spectroscopy	Elemental composition	[46]	

Transmission electron microscopy	Density Phase-contrast Diffraction	[45]	[28]
Scanning electron microscopy (SEM)	Density and topography imaging Elemental composition	[117]	
Focused ion beam (FIB)-SEM Serial Surface View	Stack of 2D SEM images		[72]
Atomic force microscopy	Topography and stiffness mapping	[118]	
Wide-angle X-ray diffraction	Crystallographic structure; texture	[119]	[120]
Small-angle X-ray scattering	Mineral size and orientation Interfaces	[121]	[122]
X-ray fluorescence	Elemental composition	[123]	
Raman spectroscopy*	Chemical composition Matrix and mineral characterization	[115]	
FTIR spectroscopy	Chemical composition Matrix and mineral characterization	[121]	
Microradiography µ-Computed tomography*	Attenuation-based contrast		[122]
Acoustic microscopy*	Material density and stiffness distribution	[123]	
Light microscopy* Confocal, phase-contrast	Structures labeling Interfaces		[47]
Nuclear magnetic resonance spectroscopy*	Molecular interactions	[29]	

Subject categories

Physical sciences / Materials science / Biomaterials / Biomineralization [URI /639/301/54/991]

Physical sciences / Materials science / Techniques and instrumentation / Characterization and analytical techniques

[URI /639/301/930/12]