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Review

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Physiological and pathological/ectopic mineralization: from composition to microstructure

Yuqing Mu[1,2,3], Wendong Gao[2,3], Yinghong Zhou[2,4], Lan Xiao[2,3], Yin Xiao[1,2,3]

[1]School of Medicine and Dentistry & Menzies Health Institute Queensland, Griffith University (GU), Gold Coast, QLD 4222, Australia.
[2]The Australia-China Centre for Tissue Engineering and Regenerative Medicine (ACCTERM), Queensland University of Technology, Brisbane, QLD 4000, Australia.
[3]School of Mechanical, Medical and Process Engineering, Centre for Biomedical Technologies, Queensland University of Technology, Brisbane, QLD 4000, Australia.
[4]School of Dentistry, the University of Queensland, Brisbane, QLD 4006, Australia.

Correspondence to: Prof. Yin Xiao, School of Medicine and Dentistry & Menzies Health Institute Queensland, Griffith University (GU), Gold Coast, QLD 4222, Australia. E-mail: yin.xiao@griffith.edu.au

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Abstract

Biomineralization is a process that leads to the formation of hierarchically arranged structures in mineralized tissues, such as bone and teeth. Extensive research has been conducted on the crystals in bones and teeth, with the aim of understanding the underlying mechanisms of the mineralization process. Pathological/ectopic mineralization, such as kidney stones, calcific tendinitis, and skeletal fluorosis, shares some similar features but different mechanisms to physiological mineralization. A better understanding will provide new perspectives for treating pathological/ectopic mineralization-related diseases. This review provides an overview of the mechanisms of the crystallization and growth of crystals in physiological and pathological conditions from a chemistry perspective. By linking the microstructures and functions of crystals formed in both conditions, potential approaches are proposed to treat pathological/ectopic mineralization-related diseases.

Keywords: Physiological mineralization, pathological/ectopic mineralization, apatite crystals, mineral crystallinity, dental tissues, bone remodeling

INTRODUCTION

Biomineralization, an emerging interdisciplinary field, deals with the formation, structure, and mechanical strength of naturally formed mineralized tissues[1]. The skeleton of animals provides mechanical support to counteract gravitational forces on land and hydrostatic pressure in the depths of the oceans. As a highly complex and exquisitely organized organ, the skeleton is structurally, and hence mechanically, heterogeneous owing to spatial distributions in the shape, size, and composition of its constituent building blocks, the mineralized collagen fibrils[2].

Nature produces a diverse assortment of mineralized structures with a high degree of complexity. These hierarchical structures exhibit superior mechanical strength[3-5] and are, therefore, of great interest to researchers in the disciplines of biotechnology and biomedical engineering. Biomineralization on a template of organic molecules is used by many organisms to produce inorganic-organic nanocomposites that result in highly ordered multifunctional materials. The biosynthesis of bacterial magnetosome[6], eggshell[7], molluscan shell[8], dental structures[9], and skeletal system[10] are all examples of biologically controlled mineral formation through organic/inorganic recognition and interaction. In the skeleton, for example, the organic matrix consists primarily of the fibrous protein collagen and around 10% of other non-collagenous proteins (NCPs)[1,11] [Figure 1]. The inorganic phase is composed of tightly packed nanocrystals made of calcium phosphates (CaPs) with the incorporation of a number of essential trace elements.

In the field of biomaterial development, the *ex vivo* bioactivity of the material is often predicted by examining the formation of an apatite layer on its surface in a simulated body fluid (SBF)[12]. Notably, slight differences in the SAED patterns of SBF-originated apatite on a Titanium substrate and bone apatite resulted from the random and ordered orientations, respectively, of apatite crystals [Figure 2][12-14]. However, there are other differences between SBF-derived apatite and bone apatite in terms of possible defects on the lattice of bone apatite due to the incorporation of trace elements in the body and crystal sizes affected by cells and cell-secreted bio-factors[15,16].

Calcification has been increasingly recognized as an important component to fully understand the pathology of some diseases. For instance, the types of breast cancer have been shown to be related to the properties of calcification in the breast[17-19]. The utilization of advanced characterization techniques within the field of material science has facilitated significant advancements in our comprehension of the formation of pathological crystals, such as X-ray diffraction (XRD), spectroscopic, and electron microscopic techniques[20,21]. By comparing to crystals in physiological mineralization, understanding the characteristics and behaviors of crystals involved in pathological or ectopic calcification can offer valuable insights into the underlying mechanisms of calcification-related disorders. This knowledge may enable the development of novel disease management strategies.

Physiological mineralization is a complex process that is essential for the development of well-organized structures in bone and teeth[22]. The intricate process occurs only in specific regions[23,24]. The regulation of physiological mineralization is well-coordinated, involving both inhibitory and stimulatory factors. Some proteins, including osteopontin (OPN), matrix Gla protein, and pyrophosphate (PPi), have been identified as inhibitors of mineralization[22,25]. In contrast, other factors, such as matrix vesicles that contain calcium (Ca) and inorganic phosphate (Pi), apoptotic bodies, and tissue non-specific alkaline phosphatase, have been shown to facilitate the initiation of mineralization[26-28].

On the other hand, pathological/ectopic mineralization occurs in soft tissues and is associated with disease conditions or medical conditions, such as injury, inflammation, and aging, causing significant morbidity

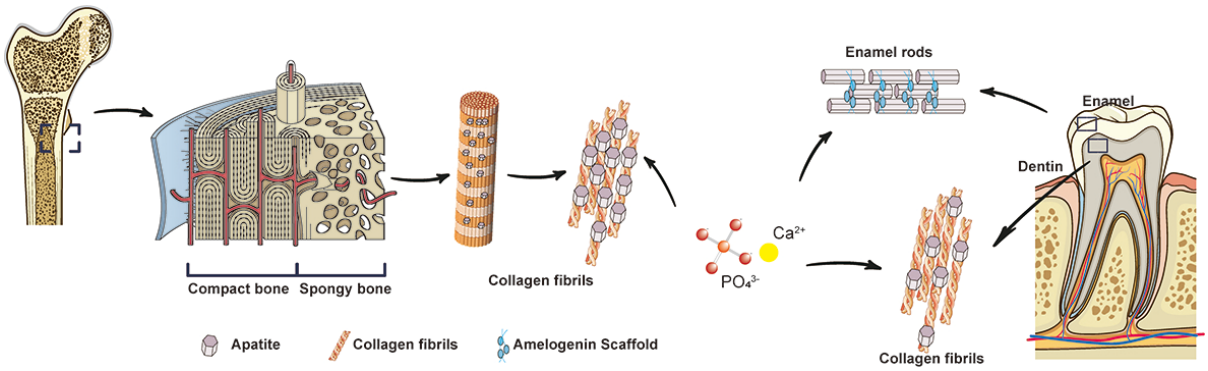

Figure 1. Physiological mineralization, mainly in bone and teeth, forms well-organized structures.

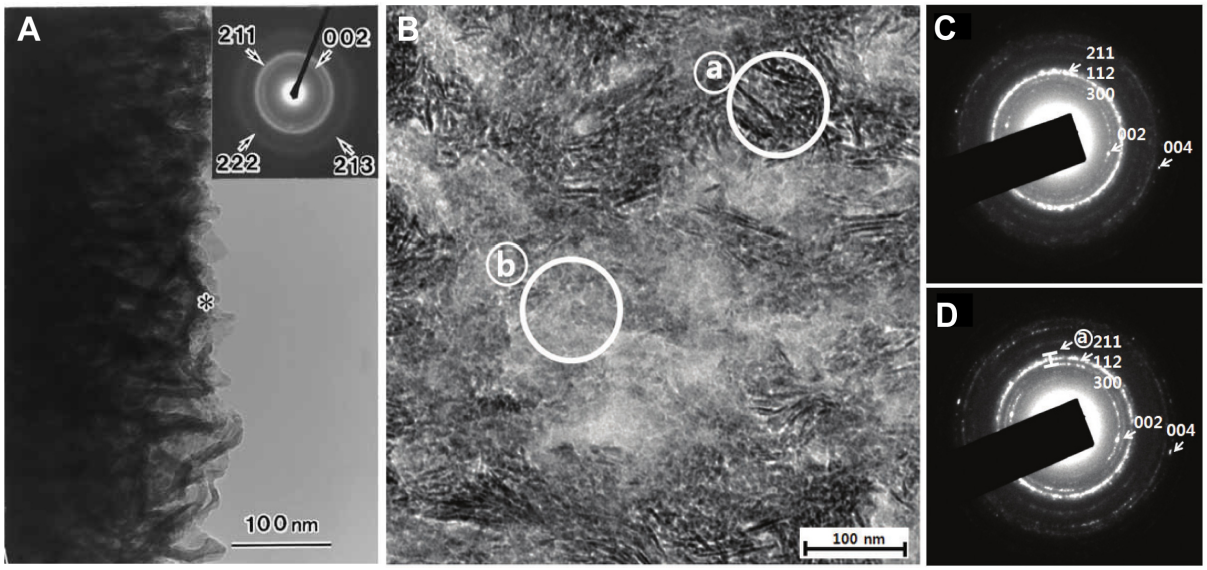

Figure 2. Transmission electron microscope (TEM) photographs of (A) SBF-originated apatite crystals on a Titanium substrate (inset: SAED pattern) (Reproduced with permission from Takadama *et al.*[14]. Copyright 2001, John Wiley & Sons), (B-D) apatite crystals from mouse femur and their SAED patterns (Reproduced with permission from Taehoon *et al.*[13]. Copyright 2010, SpringerOpen).

and mortality[29-32]. Such mineralization consists of different Ca-containing minerals, including CaPs, calcium carbonates ($CaCO_3$), and calcium oxalates (CaO_x)[20,31].

The current treatment of pathological mineralization in soft tissues includes reduced intake of Ca or reduced precipitation of Ca-containing complex. However, there are still no specific and effective treatments to prevent or counteract the condition[32,33]. Pathological/ectopic mineralization is less understood than physiological mineralization, but the commonalities between physiological and pathological/ectopic mineralization have gradually been recognized over the years[34-36]. There are still ongoing debates regarding the chemical compositions and crystal structures of pathological crystals, the dynamics of ion transport, the participation of cellular activities, the interactions of pathological crystals with surrounding tissues, and how these contribute to disease progression[36]. The compositional and structural analysis of pathological mineralization would benefit the development of better disease management through advanced treatment and prevention. In this review, bone and teeth mineralization and crystal structures are recognized as the fundamental and physiological processes in biomineralization.

The comparison is made to unveil the similarities and differences in both physiological and pathological/ectopic mineralization. In addition, current approaches, with a focus on modulating crystal formation and growth on the progression of pathological mineralization, are discussed.

OVERVIEW OF THE PHYSIOLOGICAL MINERALIZATION PROCESS

The mineralization of self-assembled organic matrices results in the formation of hard tissues in the body, and its level (or the proportion of mineral contents in the tissue) varies from around 65% in the bone to close to 100% in dental enamel [Figure 1][37,38]. The fundamental form of both bone and teeth crystals is apatite. Its compositions and structures can be modified by accommodating a wide range of ion substitutions via cell uptake[39,40]. The investigation regarding the nucleation process of apatite in solution started several decades ago, attempting to unveil the mystery of mineralization[41-45]. Along with the emergence of edge-cutting equipment and the accumulation of knowledge over the years, a multi-stage nucleation process of apatite in aqueous solution was demonstrated to start with the aggregation of charged pre-nucleation complexes, $[Ca(HPO_4)_3]^{4-}$ (Ca/P(calcium/phosphorus) = 0.3-0.4)[46]. After the formation of ribbon-like calcium-deficient o phosphates (OCPs), $[\sim Ca_6(HPO_4)_4(PO_4)_2^{2-}]$ (Ca/P = 1.0) and elongated plate-like OCPs, $[Ca_8(HPO_4)_2(PO_4)_4]$ (Ca/P = 1.33) from amorphous calcium phosphates (ACPs), apatite is finally generated[46]. It has been suggested that the evolution of the OCP structure to apatite occurred through the elimination of the hydrated shell on the surface of the nanocrystalline[37,47]. The hydrated shell on the surface of freshly precipitated apatite contains exchangeable ionic species and some proteins[37,48]. During the maturation of apatite in the solution, ions in the hydrated shell are easily exchanged, accompanied by proteins within the shell, and it leads to reduced surface reactivity and increasingly stable apatite[37,49]. This suggests that metals (such as Ca, magnesium (Mg), sodium (Na), potassium (K), *etc.*), and possibly other ions are incorporated structurally into the collagenous matrices of calcified tissues[50]. In earlier investigations of the composition of trace elements in human cortical bone and beef tendons, researchers have documented a regularity in the appearance of certain trace metals, namely copper (Cu), iron (Fe), and zinc (Zn)[23,51,52]. Additionally, non-metal trace element, e.g., fluoride (F), has also been physio-chemically linked with the bone mineral matrix[53].

Mineralization of bone

A similar crystal formation mechanism was proposed in bone based on the similarity of XRD patterns of bone mineral and crystal formed from the ACP in an aqueous solution[41-43,45]. Bone minerals can be modeled as carbonate hydroxyapatite (CHAp) with a wide range of substitutions of hydroxide (OH^-) and phosphate (PO_4^{3-}) by carbonate (CO_3^{2-}) on the lattice and the substitutions of Ca^{2+} by other metal ions, such as strontium (Sr^{2+}), Mg^{2+}, *etc.* [Figure 3][49,54]. Its composition can vary depending on species, location, diet, sex, age, and pathological conditions[37,55-57]. The substitution of carbonate results in the contraction of the *a*-axis and the expansion of the *c*-axis of the unit cell, as well as a decrease in crystallinity[58]. The presence of vacancies on the apatite crystals results in lower binding energies, thus, more soluble than stoichiometric hydroxyapatite (HAp)[25,59,60]. During the formation of bone minerals, ACP nanospheres formed on the site of bone formation or supplemented through blood are delivered and deposited within the collagen matrix by cells and further transformed into plate-like apatite via intermediates that resemble OCPs[44,61-63]. While Crane *et al.* (2006) suggest this possibility, the finding remains unreplicated and is not widely accepted[63]. However, recent evidence has shown the presence of OCPs in bone, specifically in combination with the protein osteocalcin[64]. This suggests that OCPs may play a role in bone mineral formation, although further research is needed to fully understand this process. Moreover, bone mineralization is much more complicated with the involvement of extensive biological additives, acting as promoters or inhibitors, such as proteins, trace ions, and some small organic molecules[42,65].

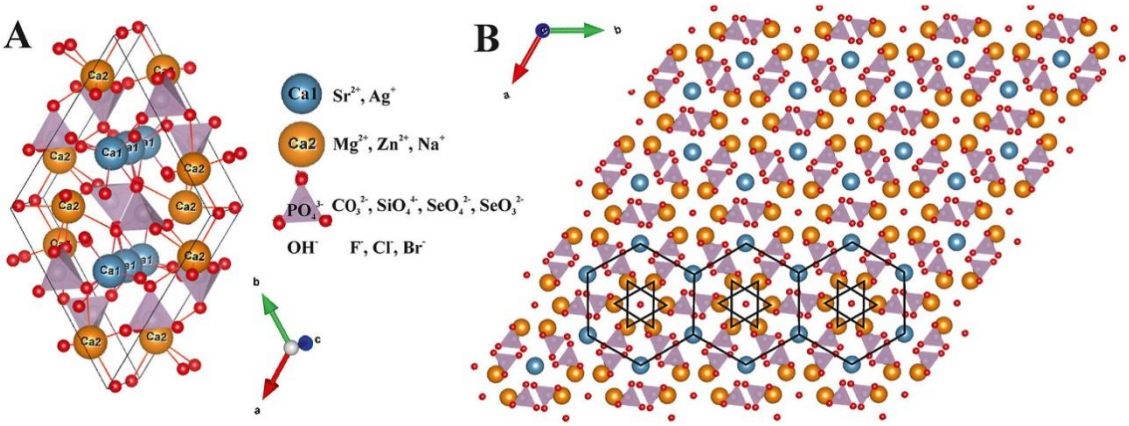

Figure 3. A schematic illustration of the crystal lattice of bone apatite (Reproduced with permission from Ressler *et al.*[54]. Copyright 2021, Elsevier). (A) the unit cell of bone apatite; (B) the top view of the lattice of bone apatite.

Based on the discovery and transformation pathway from ACPs to apatite during bone mineralization, additives are believed to get involved by preventing unstable amorphous phases from crystallizing or dissolving before or during transport to the site of interest[42]. It is also known as the polymer-induced liquid precursor (PILP) process. Numerous studies aimed at identifying influencing factors in the process over the years. For example, many NCPs found in bone growth regions regulate mineralization with acidic amino acid groups through the ability to bind Ca^{2+}, as well as high affinity for collagen fibrils, such as osteonectin and various phosphoproteins[66-68]. Organic Pi or polyphosphates, as Pi sources, participate in and affect the crystallization process of ACPs in the form of orthophosphates after being digested by alkaline phosphatase[32,69,70]. PPi, on the other hand, is a well-known inhibitor of apatite crystal formation[71].

Mineralization of teeth

In general, teeth mineral [$Ca_{(10-x)}Na_x(PO_4)_{(6-y)}(CO_3)(OH)_{(2-z)}F_z$] (x, cation substitutions; y, carbonate substitutions; z, fluorine substitutions) varies from both stoichiometric HAp and bone apatite[40]. Its chemical composition, the mineralization level of the organic matrix, and crystal size and orientation are tailored to meet different mechanical needs at different locations[37,72,73]. Enamel and dentin are two kinds of hard tissues in teeth. Dentin is capped by enamel and, in the root, is covered by cementum.

Dentin is less mineralized and shares a similar mineralization pattern with bone, namely matrix-mediated mineralization[74,75]. It is formed by the mineralization of the dentin matrix (composed of type I collagen), mediated by some non-collagenous matrix proteins (NCPs), such as dentin phosphoprotein (DPP), dentin sialoprotein (DSP), and dentin matrix protein-1 (DMP-1)[76]. However, unlike bone, little or no remodeling takes place in dentin. Compared to enamel, dentin has a higher capacity for F uptake through systemic ingestion due to its less crystallinity and accumulation through life[77].

Enamel is the hardest tissue in the vertebrate body, composed of crystalline HAp[78]. In contrast to the bone, which uses collagen as the substrate for mineralization and goes through remodeling throughout its lifetime, enamel does not contain collagen and does not remodel[76,79]. During enamel mineralization, the synthesized protein mixture assembles to form a matrix that regulates the precipitation of HAp[80,81]. Once the full thickness of enamel has been formed, HAp crystals expand slowly into the space previously occupied by matrix proteins and water[82]. Mature HAp crystals in enamel are ribbon-like fluoridated carbonate HAp, 50-70 nm in width and 20-25 nm in thickness[83].

Cementum is a thin layer that covers the entire root surface and anchors the roots of teeth to the jaw. Similar to the bone, it is composed of water, an organic matrix (mainly collagen, glycoproteins, and proteoglycans), and minerals and is formed through the formation of HAp in deposited type I collagen by cementoblasts[84-86]. Compared to dentin (70%) and enamel (> 95%), cementum (40%-50%) is less mineralized[86]. In addition to HAp, small amounts of ACPs were also found in cementum[85]. The presence of the amorphous phase results in a greater capacity of cementum for the adsorption of F and other elements over time and readily decalcification in acidic environments[85]. For example, cementum contains F up to 0.9% ash weight and increases with age[87]. Regulation of teeth mineralization is, in part, regulated by the ratio of Pi and PPi, as well as other factors, such as genetic modifications, age, diet, oral hygiene, and certain diseases, such as periodontal diseases, caries, root resorption, tumoral lesions, or trauma[88,89].

Otoconia

Otoconia, which is composed of $CaCO_3$, is found in the inner ear of humans and other vertebrates. They are positioned to sense stimuli in directions and send signals to the brain to maintain bodily balance[90]. Similar to the bone, the organic matrix in otoconia determines the orientation, sizes, and shapes of crystals by providing a framework[91]. Additionally, otoconia contain high levels of Ca, Na, Mg, K, P, sulfur (S), and chloride (Cl)[90]. However, the underlying molecular etiology remains unknown, neither the functions of otoconial proteins nor the crystal formation.

PATHOLOGICAL (ECTOPIC) MINERALIZATION IN MAJOR ORGANS

Physiological mineralization is restricted within the skeletal system, including bone, teeth, and calcified cartilage[92]. In contrast, pathological (ectopic) mineralization can occur not only in the skeletal system but also in soft tissues, such as the breast, blood vessels, kidney, pancreas, and prostate, mostly composed of CaPs, $CaCO_3$, and CaO_x [Figure 4][22,31]. There are three structural types of deposits observed in ectopic calcification, single crystals, polycrystalline deposits, and calcified matrix. The specific type and characteristics of the deposits can vary, depending on the tissue and the underlying mechanisms. For example, single crystals are typically small and uniform in size and often are found in tendons and ligaments[93,94]. The formation of single crystals can be initiated by the presence of specific proteins or molecules that act as nucleation sites. Polycrystalline deposits, on the other hand, consist of multiple small crystals randomly arranged in tissues, such as blood vessels and heart valves[95]. In contrast, the calcified matrix consists of alternating layers of mineralized and non-mineralized tissues and can be found in a variety of tissues, including cartilage and blood vessels[96]. Pathological/ectopic calcification occurs in both genetic and acquired clinical conditions and affects the prognosis of diseases[32,97]. Pathological/ectopic calcification can occur through different mechanisms, depending on the involvement of cells, including cell-induced, cell-controlled, or spontaneously precipitated. For example, vascular calcification can be triggered by damage to the endothelial cells lining the blood vessels, leading to the recruitment and differentiation of smooth muscle cells into osteoblastic cells that actively deposit Ca and Pi[35]. Cell-controlled calcification occurs with specific mineralization-related factors released by cells in response to injury, inflammation, or bacterial infection[98,99]. Spontaneous deposition may happen as a result of changes in the environment without the involvement of cells, such as oxidative stress, local pH, and the supersaturation of ions[100,101]. Understanding the formation, structure, and composition of crystals deposited in the physiological and pathological conditions will aid the development of therapeutic strategies to prevent and/or regulate pathological/ectopic calcification[31,102] [Table 1].

Bone

Bone tissue undergoes continuous remodeling to maintain its density and strength in a stable state. The bone mineralization process is tightly regulated by osteoblasts (bone-forming cells) and osteoclasts (bone resorption cells). Osteocytes, which are embedded in the bone matrix, also play important roles in

Table 1. A summary of pathological/ectopic mineralization in terms of the composition and the involvement of cells

Tissue	Composition	Involvement of cellular activities	References
Bone	HAp	Cell-induced	[112]
Joint	CPPD, HAp, TCP, OCP, whitlockite, sodium urate	Cell-derived articular cartilage vesicles	[120,123,124,237]
Tendon	Carbonate HAp	Cell-induced	[94,128,130-132]
Teeth	Brushite, dicalcium phosphate dihydrate, OCP, HAp, whitlockite	Cell-mediated	[134,135]
Salivary gland	HAp, whitlockite, brushite, OCP	*Unknown*	[137-139]
Heart and blood vessel	HAp, whitlockite	Cell-induced	[20,35,140-142]
Kidney	CaOx, CaP, the mixture of struvite magnesium ammonium phosphate, carbonate HAp	Cell-mediated	[151,153-155]
Brain	HAp	*Unknown*	[171,172]
Ocular	Whitlockite, HAp	*Unknown*	[20,21]
Breast	CaPs (carbonate HAp and whitlockite), CaO_x	Cell-induced	[182-185]
Pancreas	$CaCO_3$	*Unknown*	[188,189]
Prostate	Carbonate HAp, whitlockite, CaO_x,	Cell-mediated	[191,192,238]
Placental	CaPs	Cell-mediated	[194,195]
Lymph nodes	HAp, whitlockite	*Unknown*	[197,198]

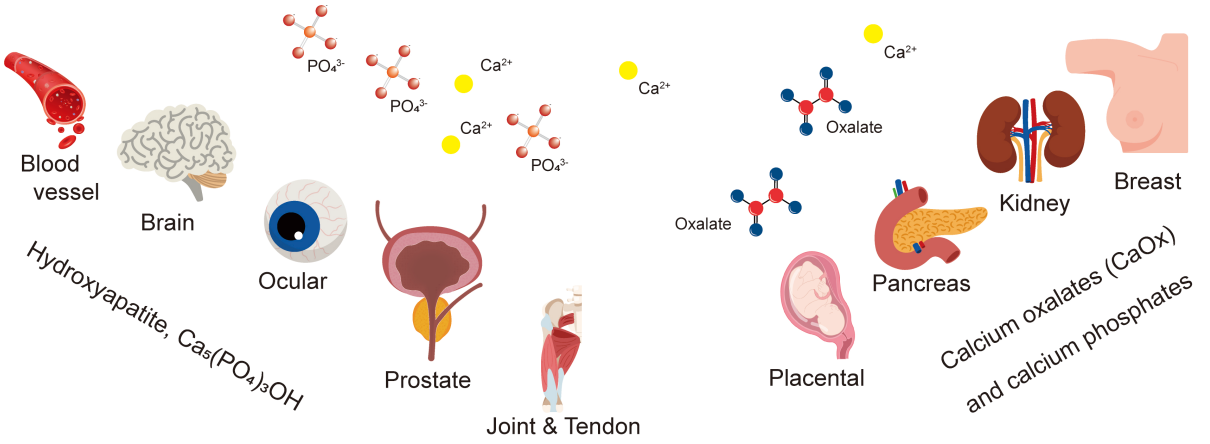

Figure 4. Pathological mineralization in soft tissues (blood vessels, brain, ocular, prostate, joint & tendon, placental, pancreas, kidney, and breast). The major forms of pathological/ectopic mineralization are calcium phosphates, calcium carbonate, and calcium oxalates.

regulating the bone remodeling process. The disruption in the balance between bone resorption and bone formation resulting from several pathological cues will affect bone health, metabolism, or homeostasis and lead to irregular remodeling activity and pathological bone mineralization[103]. Pathological bone mineralization can be roughly categorized as hypomineralization, hypermineralization, and other abnormal mineralization patterns.

Hypomineralization

Hypomineralization refers to insufficient mineralization of the bone matrix, resulting in reduced bone density and increased fracture risk[104]. Some several factors and conditions contribute to hypomineralization[105-107]. Rickets and osteomalacia are examples of pathological hypomineralization conditions[103]. Rickets, a disorder affecting children, and osteomalacia, its adult counterpart, can be caused by insufficient intake of essential nutrients, such as calcium and vitamin D, and hereditary factors[103]. The

deficiency of calcium and vitamin D can result in a reduction in Ca that leads to impaired bone mineralization and a higher proportion of organic matrix compared to inorganic mineral content, which contributes to reduced mechanical strength and increased fracture risk[105].

Besides, certain genetic disorders can induce abnormal bone development, such as osteogenesis imperfecta, a condition characterized by fragile bones due to the mutation in genes responsible for collagen production that leads to aberrant collagen production, and hypophosphatasia, an inherited disorder that results from a deficiency in alkaline phosphatase, which is essential for proper bone mineralization[106,108].

Hypermineralization

Hypermineralization, characterized by abnormally high bone mineral density (BMD), is associated with hereditary and nonhereditary disorders[104]. For example, in hereditary disorders, such as osteopetrosis and osteosclerosis, a dramatic decrease in osteoclast number or osteoclast activity and increased bone mass and density were observed. At the crystal level, low remodeling activities alter the crystal size/shape and increase the amount of highly mineralized bone tissues, leading to decreased toughness of bone tissues and thus increasing the risk of fracture[104]. Skeletal fluorosis is another nonhereditary disorder associated with excessive ingestion of F. The accumulation of F results in abnormal bone deposition and adsorption. At the crystal level, the hydroxyl group in HAp is substituted by F, resulting in an increased crystallinity, crystal size, and elastic modulus[109].

Other abnormal mineralization patterns

Osteoporosis, one of the most prevalent pathological abnormal mineralization conditions, is mainly related to menopause and aging[104,110,111]. Osteoporosis is diagnosed based on low BMD and leads to reduced bone mass and deterioration in bone micro-architecture, which further increases the susceptibility to fracture[110]. In osteoporosis, bone resorption exceeds bone formation, resulting in altered mineralization properties, including mineral content, composition, and crystal size, which leads to reduced fracture resistance[110]. It has been reported that the bone tissue Ca/P ratio was reduced in osteoporosis patients and the imbalance increased the defect in the HAp network, thus resulting in a less rigid HAp crystal structure and further contributing to the increased fracture susceptibility in osteoporotic bones[112]. Meanwhile, the uneven distribution of minerals in the bone matrix is observed in some pathological conditions, such as Paget's disease, which is a chronic disorder that the normal bone remodeling process is disrupted, leading to abnormally shaped, enlarged, and weakened bones that are more susceptible to fractures and deformities.

The presence of whitlockite in osteocyte lacunae (micropetrosis) is associated with pathological conditions, as it is primarily found in the pathological mineralization of various soft tissues, dental calculus, and occasionally in enamel and dentine[113]. Notably, Mg-whitlockite [$Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$] has been detected in post-apoptotic osteocyte lacunae in human alveolar bone, but this unusual mineral has not been found in the extracellular matrix of mammalian bone under normal conditions[113]. The notion that Mg-whitlockite is a significant constituent of bone minerals remains unsubstantiated, and it is considered a pathological biomineral[114]. Therefore, contrary to some claims, biomaterials containing Mg-whitlockite do not represent a bioinspired or biomimetic approach to bone repair[113].

Joint

Cartilage calcification that has been observed in the hip and knee is pathological in almost all osteoarthritis (OA) patients[115]. The deposition of calcium pyrophosphate dihydrate (CPPD) has been found in patients suffering from acute attacks of pseudogout in the joints[116-118]. Other CaPs have also been found in synovial fluids, synovium, and cartilage of OA patients, such as carbonate HAp, tricalcium phosphate (TCP), and

OCP[116,117,119,120]. Articular cartilage calcification that occurs in end-stage OA shows the accumulation of predominant HAp crystals[116,121]. The deposition of HAp initiates from articular cartilage vesicles (ACVs) and induces stress in articular chondrocytes, which ultimately results in the phenotypic change of chondrocytes and the formation of more HAp crystals[121,122]. Besides, whitlockite, another kind of CaP-based mineral, has also been found in the mineral phase of osteoarthritic articular cartilage and intervertebral disc[123,124]. Under certain circumstances, such as elevated Mg concentrations, acidic microenvironments, and the presence of specific proteins or organic molecules, Mg partly substitutes Ca, inhibits apatite originating from ACP, and aids the precipitation of whitlockite.

Tendon

Tendon mineralization (calcific tendinitis) is the cell-mediated calcification of living tissues that causes joint pain[94]. It typically affects the shoulder and hip, as well as other sites, such as the hand and wrist, foot and ankle, and neck[125]. Four well-defined phases of calcific tendinitis are formative (pre-calcific), calcific, resorptive, and reparative[94,126]. In the formative phase, a portion of the tendon undergoes a fibrocartilaginous transformation. During the calcific phase, calcium crystals are deposited in small vascular structures located between collagen fibrils[126,127]. Followed by calcification, the appearance of thin-walled vascular channels at the periphery of the calcium deposits marks the initiation of the resorptive phase, which involves Ca removal by macrophages and multinucleated giant cells[128]. Computed tomography studies showed the porous structure of calcific deposits throughout the tendon[129]. Structural and compositional analysis revealed that the chemical composition of mineralized deposits in calcific tendinitis is poorly crystallized carbonate HAp, having a Ca/P molar ratio ranging between 0.9 and 1.5[128,130-132].

Teeth

Pathological mineralization on teeth results from a variety of factors, such as changes in the oral environment, genetic predisposition, or underlying medical conditions[88,89]. In dentin, pathological mineralization happens in the form of dentinogenesis imperfecta, causing weakened teeth that are prone to cavities and fractures[133]. Dental calculus builds up on teeth (both supragingival and subgingival), is composed of inorganic components (brushite, dicalcium phosphate dihydrate, OCP, HAp, and whitlockite) and salivary proteins adsorbed on the tooth surface[134,135]. The level of dental calculus is affected by oral hygiene habits, diet, age, systemic diseases, and medications[135]. In enamel, pathological mineralization appears as spots on the surface of the tooth. It can be caused by malnutrition, high fever during childhood, or exposure to excessive F[77].

Salivary gland

A salivary gland stone (salivary calculi or sialolithiasis) found in the salivary gland consists of a central organic core and a layered cortex of inorganic components[136]. The crystals found in the salivary gland were randomly oriented carbonate HAp, whitlockite, and less frequently brushite and OCPs[137-139].

Heart and blood vessels

Cardiovascular calcification, the deposition of apatite and whitlockite, is closely associated with increased risks of several mortal diseases, such as blood vessel stenosis, ischemia, stroke, and heart disease[20,35,140-142]. Based on its histological appearance, the calcification can be distinguished as amorphous (lacking tissue architecture) and chondro-osseous (having the tissue architecture of cartilage or bone)[143].

Calcification that occurs in the intimal layer of the arteries links to arterial obstruction and atherosclerotic plaque rupture, leading to stroke or myocardial infarction[35,144]. Calcification that occurs in the medial layer, also known as Monckeberg's medial sclerosis, is associated with vessel stiffness, systolic hypertension, and progressively increased risks of diastolic dysfunction and heart failure[35,145,146]. Even though the mechanism

of cardiovascular calcification is not fully understood, it is no longer acknowledged as a passive accumulation of minerals but an active inflammatory and/or osteogenesis-related signaling process[147,148]. Several influencing factors have also been identified in the past few decades, such as the loss of inhibition, induction of osteogenesis, accumulation of nuclei, and cell death[98]. For example, cell-secreted small membrane-bound microparticles, such as apoptotic bodies and matrix vesicles, showed their capacities to concentrate Ca and Pi and initiate crystal nucleation in response to inflammatory stimuli[100,144]. Additionally, lipids and cytokines released by macrophages were also found to accelerate osteogenic differentiation and calcification of vascular smooth muscle cells (VSMCs)[100,101].

Kidney

Kidney stones are one of the most common and painful urinary disorders[149]. In most cases, they result from the nucleation and aggregation/growth of crystals from supersaturated urine [Figure 5][150,151]. Based on their composition and pathogenesis, kidney stones are commonly classified into five types: (a) Ca-containing crystals; (b) struvite or magnesium ammonium phosphate stones; (c) uric acid stones or urate; (d) cystine stones; and (e) drug-induced stones[152].

Ca-containing stones are predominant renal stones, typically composed of CaO_x (50%), CaP (5%), or a mixture of both (45%)[153]. CaO_x exists in the form of CaO_x monohydrate (COM, or whewellite), CaO_x dihydrate (COD, or weddellite), or a mixture of both forms[151,154]. Struvite stones in the body are shown to be a mixture of struvite magnesium ammonium phosphate [$MgNH_4PO_4 \cdot 6H_2O$] and carbonate HAp[155]. In addition to the most common Ca-containing stone that comprises 80% of urinary calculi, struvite accounts for 10%-15%, and uric acid stones account for 9%, leaving 1% of the rest of the stone types, including cystine stones and drug-induced stones[152,156,157].

The pathogenesis of kidney stones is a multi-step process, including crystal nucleation, aggregation, and retention. The nucleation of calculus occurs in a supersaturated liquid, where Ca and oxalate combine to form insoluble micro-clusters or start with existing nucleation centers [Figure 5][150,152,157]. Crystal aggregation and secondary nucleation of crystals account for crystal growth and interactions between crystals and cells and extracellular matrix[158]. The formation of kidney stones is affected by the supersaturation of Ca and oxalate in the urine, urinary pH, and some molecular modulators in the environment[157,159,160]. For instance, the growth of CaO_x crystals is associated with a urinary pH range of 5.0 to 6.5; uric acid stones form at low urinary pH (pH < 5.05), whereas CaP and struvite favor a pH range over 7[152]. In addition to factors that affect the formation of kidney stones by altering the environment, there are several modulators actively modulating the nucleation and aggregation of pathological crystals. For example, PPi and citrate reduce the nucleation and growth of Ca-containing crystals, as well as Mg[157,160,161]; Biomolecules, such as OPN, prothrombin F1 fragment, and calgranulin, have been shown to inhibit the crystallization of CaO_x and CaP by binding to Ca and hindering crystal growth[157,162-164].

Brain

Brain mineralization occurs in the basal ganglia and other regions, such as the cerebellum, thalamus, and brainstem[165,166]. Patients presenting brain calcification exhibit impaired motor and cognition, such as dystonia, parkinsonism, psychosis, and dementia[165,167,168]. It is associated with neurological or metabolic disorders (secondary hypoparathyroidism and mitochondrial diseases) and other acquired conditions, such as injuries (infection, ischemia, and trauma) and toxicity related to manganese (Mn), Fe, lead (Pb), carbon monoxide, and normal aging[165,169,170]. The major composition of brain mineralization is HAp[171,172]. Other components, such as Zn, Fe, and Mg, are also present in the deposits[173]. However, currently, there is no established method to determine whether it is a passive process, which can be attributed to normal aging, or an active process mediated by cellular activities[171,172].

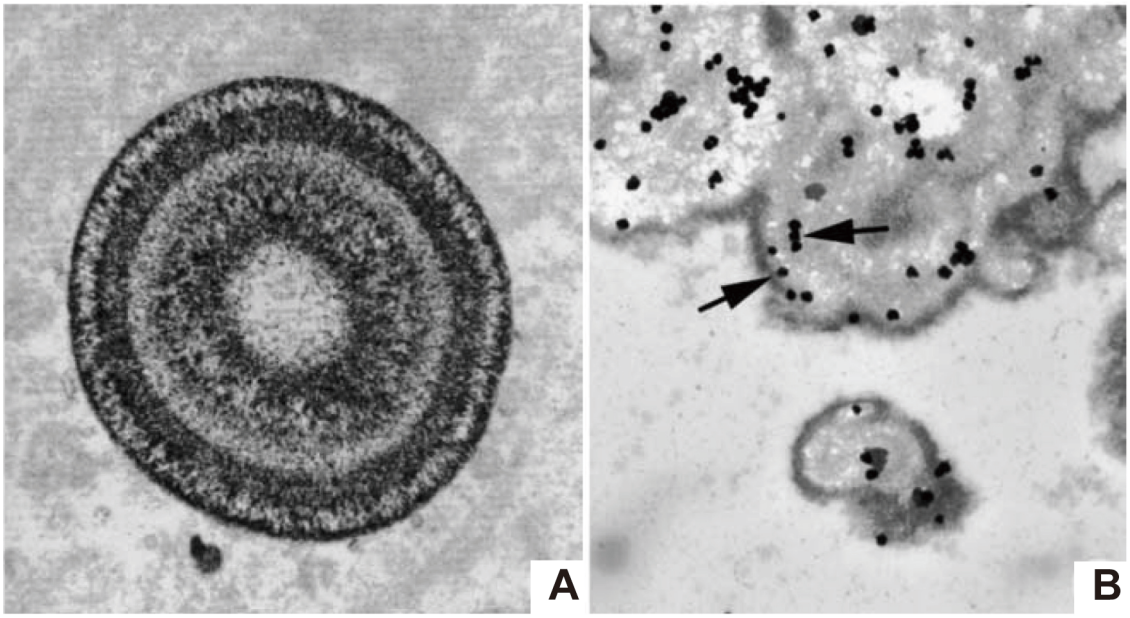

Figure 5. Crystal deposition in the urinary system (Reproduced with permission from Evan *et al.*[150]. Copyright 2005, Elsevier). (A) The initial sites of the deposition of kidney stones in transmission electron microscopic images and (B) immunogold staining showed the localization of osteopontin in the plaque.

Ocular

Ocular mineralization can be found in the cornea, retina, optic nerve, and Bruch's membrane[92,174-176]. In the aging eye, the accumulation of protein- and lipid-containing deposits external to the retinal pigment epithelium (RPE) can lead to macular degradation and, consequently, blindness[92,177]. Three types of structures of minerals have been found in age-related macular degradation, spherules (whitlockite), plaques (amorphous apatite), and nodules (apatite)[20,21]. However, the mechanism of the formation of calcified nodules remains unknown.

Breast

Breast mineralization, also called microcalcification, has been suggested to be a consequence of either injury or diseases, such as chronic kidney disease, hypertension, or metabolic syndrome[178,179]. The formation of microcalcification is related to the acquisition of mesenchymal characteristics in breast cancer cells, affected by transforming growth factor beta (TGF-β) or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[180,181]. Multiple phases of breast microcalcifications have been identified: CaPs (such as carbonate HAp and whitlockite), amorphous CaP, and, less commonly, CaO_x[182-185]. However, the exact origins of minerals remained unclear. Nevertheless, the screening and evaluation of the morphology and distribution of microcalcification aid in determining the likelihood of whether the calcifications are benign, intermediate, or necessitate further investigation[17]. For instance, CaOx (type I calcification) is detected in benign breast lesions or lobular carcinoma, whereas HAp calcification (type II calcification) is detected in both benign and malignant breast tissues[18,19]. Compared to those formed in malignant ducts, type II microcalcification formed in benign ducts was found to contain a higher amount of $CaCO_3$ and a lower amount of protein[186]. In some malignant specimens, Mg and Na have also been detected[187]. Elevated Na levels have been found in malignant specimens, but no correlation has been found between the level of Mg and malignancy[187].

Pancreas

Pancreatic stones (Pancreatic calculi) occur in the main ducts, side brunches, or parenchyma[188]. It contains an inner nidus core and outer shell, primarily constituting $CaCO_3$, in the form of calcite (major) and aragonite, besides Pi and other protein content[188,189]. The inner protein nidus contained Fe, chromium (Cr), and nickel (Ni), whereas the outer calcite shell contained Ca and 17 other elements[190].

Prostate

Prostatic calculi are usually found as apatite or whitlockite, less frequently CaO_x[191,192]. They are classified as primary/endogenous or secondary/extrinsic stones[193]. Endogenous stones are commonly caused by obstruction of the prostatic ducts or chronic inflammation; extrinsic stones are mainly caused by urine reflux[193]. Klimas *et al.* suggested two mechanisms regarding the calcification of prostatic calculi: the calcification of the *corpora amylacea* (type I calculi, composed of Na, S, P, Ca, and Zn) and the precipitation of prostatic secretions (type II calculi, composed of Ca and P)[191].

Placental

The placenta, a highly vascularized organ, mediates the communications between two circulatory systems. Placental calcification occurs when small calcium deposits build up on the placenta and is recognized as CaPs (the ratio of Ca/P = 2.00 ± 0.05) observed in placenta tissue[194,195]. It is often found in both preterm and term birth and serves as a predictor of adverse pregnancy outcomes[196]. However, the mechanisms of placental calcification are poorly understood[194].

Lymph nodes

Calcification in the lymph nodes occurs following diseases such as granulomatous infections[197]. Two patterns of calcification were found in the submandibular and neck regions: small blocks with different textures and islets surrounded by soft tissues, respectively[198]. In the submandibular specimen, apatite, together with a minor amount of whitlockite, was found[198]; In the neck specimen, whitlockite was found most frequently[198].

POSSIBLE PREVENTION AND TREATMENTS OF PATHOLOGICAL MINERALIZATION

Current treatments for the removal of calcified plaque include mechanical removal using rotary blade ablation or chemically dissolving *in situ* with acid via a catheter device[199-201]. In addition to mechanical and chemical debridement, reduced mineral ion intake from the diet or ions from bone stores have also been considered potential treatments for pathological/ectopic calcification in clinical practices[201]. Other approaches have been explored in targeting regulatory molecules in the body. For example, the monoclonal antibody Denosumab was developed to interfere with osteoclast functions and serum Ca levels, and PPi inhibited uremic vascular calcification without interfering with the mineralization of bone[202,203]. However, there are still no well-established and acknowledged treatments to prevent or counteract pathological/ectopic mineralization.

Current strategies targeting cellular mechanisms of calcification provided promising avenues for better administration of pathological mineralization in soft tissues, either reducing the nucleation of pathological crystals and crystal growth or reducing the circulating mineral ions in the serum[204]. Several endogenous calcification inhibitors have been identified, such as fetuin-A, vitamin-K dependent matrix-Gla protein (MGP), PPi, and OPN[23,33]. The serum protein fetuin-A is an important inhibitor of extra-skeletal calcification in the plasma and tissue fluids[205]. It is derived from the liver and acts as a chaperone, stabilizing excess mineral ions by forming a colloid with mineral ions or completely insoluble calciprotein particles[206-208]. MGP was the first inhibitor of artery calcification to be characterized *in vivo*[24]. It serves as an inhibitor of bone morphogenic protein-2 (BMP2), driving changes in cells toward an osteogenic-like

phenotype and subsequent calcification[209,210]. PPi is identified as the principal inhibitor of HAp deposition, evidenced by antagonizing the ability of Pi to crystalize with Ca to inhibit biomineralization and acquired clinical conditions regarding pathological calcification upon its deficiency[32]. OPN is found to be associated with nascent mineralization foci during bone mineralization and present at interfaces where mineralization is needed to be quenched[211,212].

Several approaches to regulating the metabolisms of these natural inhibitors exhibited promising results in treating pathological mineralization. For example, Vitamin K supplementation showed the effect of reducing the progression of vascular mineralization and subsequent arterial stiffness[33,213]. Bisphosphonates, derivatives of PPi, stabilize the Pi groups by a phosphorus-carbon-phosphorus backbone. Compared to PPi, which can be easily hydrolyzed, bisphosphonates have extended plasma half-life and improved stability[32]. Bisphosphonates showed their potency to reduce fractures in postmenopausal osteoporosis[214]. They have also been found to reduce soft tissue calcification and interfere with osteoclast functions[33,204]. Alendronate, a widely prescribed bisphosphonate, has shown good tolerance and improvements in several patients with brain calcification[167]. Other bisphosphonates, such as etidronate, pamidronate, and ibandronate, have been demonstrated to inhibit aortic calcification both *in vitro* and *in vivo*[202,215-218]. In addition to bisphosphonates, a few proteins associated with the metabolism of PPi have also been identified to be promising for targeted treatment of pathological mineralization, such as ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1), ATP binding cassette subfamily C member 6 (ABCC6), progressive ankylosis protein (ANK), and tissue-nonspecific alkaline phosphatase (TNAP)[219-222]. Inspired by the inhibitory effect of acidic urinary macromolecules (e.g., OPN) on the formation of CaO_x *in vitro*, polymeric carboxylic amino acids, poly-L-glutamic and aspartic acid, were found to inhibit the crystallization of CaO_x[223]. A similar inhibitory effect was also found to be achieved by acidic polyanion poly (acrylic acid)[224-226]. Additionally, sodium thiosulfate (STS) treatment was found to inhibit calcium salt precipitation in calciphylaxis[227-230]. Nevertheless, these results were limited either in the long-term efficacy or potential toxicity of the STS treatment[33].

Moreover, the presence of trace elements has been shown to play a role in the crystal formation kinetics or external morphology of a growing crystal. It could be promising in designing therapeutic approaches again pathological mineralization[102,231]. For example, Mg, Zn, aluminum (Al), Fe, and Cu are indicated as growth inhibitors of CaO_x at very low concentrations[232-235]. Adding $AlCl_3$ or $FeCl_3$ to transplanted valves effectively delayed the onset of valve calcification by blocking TNAP activity[236].

CONCLUSIONS AND FUTURE PERSPECTIVES

Our understanding of pathological/ectopic mineralization has been dramatically improved in parallel to bone biology and advanced characterization techniques widely used in the field of material science[22,92,201]. Significant progress has been made in elucidating the properties of pathological crystals and the underlying mechanisms of pathological/ectopic calcification that occurred in soft tissues over the years. By elucidating the physiological mechanisms of bone mineralization and their relationship with mineralization phenomena, we can develop targeted therapeutic interventions to prevent, manage, or treat these disorders. However, there are still some debatable questions regarding (a) the composition and structure of pathological crystals mediated by cellular activities and factors in the surrounding environment; (b) the dynamics of ion transport; (c) the involvement of cells and related cellular activities; (d) the interactions between pathological crystals and the surrounding tissues; and (e) how these contribute to disease progression[36]. Even though attempts have been made to simulate the crystallization process, it is still challenging to comprehend the *in situ* crystallization process due to the involvement of cellular activities. Nevertheless, a more comprehensive understanding of how the structures are formed, progressed, and adapted to changing needs enables us to conceive new insights into the progression of the pathological

condition and guide future therapeutic designs to prevent and manage pathological/ectopic mineralization.

DECLARATIONS

Authors' contributions

Conceptualization: Mu Y
Writing-original draft: Mu Y, Gao W
Writing-review and editing: Mu Y, Gao W, Zhou Y, Xiao L, Xiao Y

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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