

***In Situ* Mineralization Strategies in Collagen-Based Scaffolds for Bone Tissue Engineering: Mechanisms, Structure–Function Relationships, and Translational Barriers**

Maria Ambrose. A¹, Swetha. K², Usha Nandhini. S^{3*}

¹Research Scholar, Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Chennai, India.

ambrosealphonse773@gmail.com

ORCID ID: 0009-0007-4618-5118

²Research Scholar, Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Chennai, India.

swethakarunanithiswetha@gmail.com

ORCID ID: 0009-0006-9166-6853

^{3*}Assistant Professor, Department of Biotechnology, School of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, Chennai, India.

usha.biotech@sathyabama.ac.in,

ORCID ID: 0000-0003-0550-2758

***Corresponding Author:** Usha Nandhini. S, Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India. Email: usha.biotech@sathyabama.ac.in

ABSTRACT

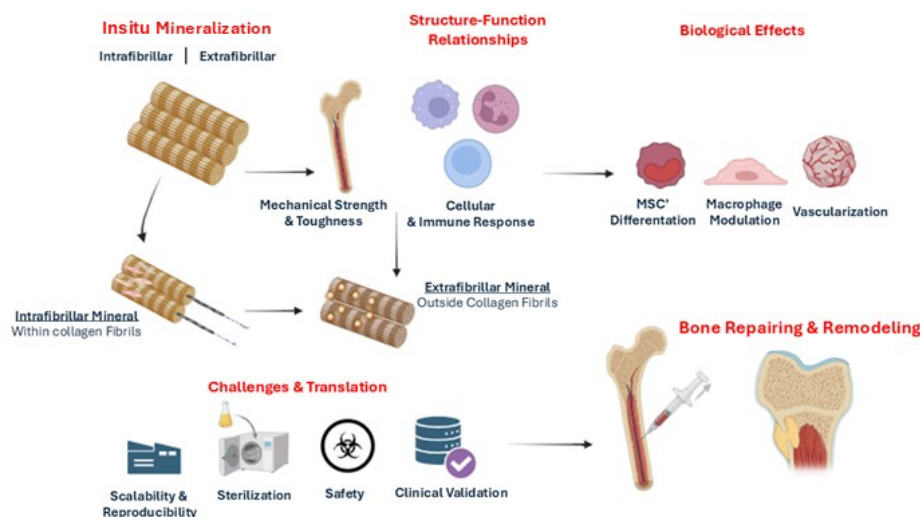
The restoration of large bone lesions is a chronic clinical issue because the existing grafts and artificial materials are not able to fully mimic the hierarchical structure and bone functions of normal bone. In bone, a mineralized collagen composite, apatite crystals are buried within and surrounding collagen fibrils. As a consequence, *in situ* mineralization techniques, where the mineral is formed inside the collagen-based scaffolds and not added as separate particles have received much attention. These techniques promise better control over the location, orientation and content of minerals and could permit biomimetic tuning of mechanical competence and biological performance. *In situ* mineralization methods in collagen-based scaffolds of bone tissue engineering are critically investigated in the study including the relationship between mechanisms of mineralization, the multiscale structure obtained and the functional outcome. First, collagen fibrils architecture and the relevant properties of the extracellular matrix of natural bone are referred to as design templates. Subsequently, the mechanisms of nucleation, the control parameters, and the balance between the intrafibrillar and extrafibrillar mineral deposition are discussed in the context of the key *in situ* mineralization pathways, including ion-mediated processes, polymer-prompted liquid precursor systems, enzyme-based methods, and stimuli-promoted methods. We assemble information on the impact of mineral distribution, crystal size and direction, hierarchical porosity on osteogenesis, mechanical properties, cell behaviour, degradation and immunological response. The manufacturing repeatability, scale-up, sterilization effects, regulatory categorization, lack of long-term *in vivo* evidence, and poor benchmarking against clinical standards are the main translational gaps that we have identified. We argue that the creation of quantitative structure-function correlations and clinically useful assessment models are currently more significant to the further development than another mineralization recipe. All said and done, *in situ* mineralized collagen scaffolds remain a possible route towards biomimetic bone regeneration; however, their clinical relevance will be determined more by addressing the reality of translation challenges rather than enhancing mineralization chemistry.

Keywords – *In situ* mineralization, Collagen scaffolds, Bone tissue engineering, Biomimetic mineralization, Hydroxyapatite.

How to cite this article: Maria Ambrose A, Swetha K, Usha Nandhini S., *In Situ* Mineralization Strategies in Collagen-Based Scaffolds for Bone Tissue Engineering: Mechanisms, Structure–Function Relationships, and Translational Barriers. *Int J Drug Deliv Technol.* 2026;16(81s): 1408-1428; DOI: 10.25258/ijddt.16.81s.175

Source of support: XX

Conflict of interest: XX



Introduction

Large bone defects represent a major clinical problem, as they surpass the natural regenerative capacity of the bone, causing chronic pain, loss of functionality and permanent disabilities [1]. Non-unions in long bones are found in 5-10% of all cases, and involve several surgical interventions, long rehabilitation period and bone grafting; nevertheless, the outcome of this treatment is often unpredictable, particularly for large or infected defects [2-4]. These diseases impose a high social and economic burden due to increased medical expenses, hospitalization and lower quality of life. Thus, there is an urgent need for development of new strategies in bone tissue engineering which employ biocompatible materials, cells and bioactive factors for bone regeneration and repair [5].

The bone is an efficient load-bearing and self-remodeling tissue, and its remarkable performance arises from its being a hierarchical composite of type I collagen, nanoscale carbonated hydroxyapatite and water, rather than a simple two-phase organic-inorganic material [6-9]. Water occupies both the pore space and the intrafibrillar/interfibrillar compartments of the collagen-apatite composite; it acts as a plasticizer and, together with bound-water content, is a major independent determinant of bone toughness, post-yield behavior and fracture

resistance, so a biomimetic scaffold that reproduces only the collagen to mineral ratio without accounting for its hydration state cannot fully recapitulate native bone mechanics [10, 11]. Collagen fibrils are arranged at the molecular level in a tough, flexible organic matrix and nanocrystalline apatite is deposited in and around the fibrils, which provides stiffness and compressive strength [6, 7]. The mineral platelets are mainly aligned with their crystallographic c-axis parallel to the collagen fibril axis and this allows the platelets to transfer stress between the organic and inorganic phase as well as to increase anisotropic mechanical properties [12, 13]. This structural motif is scale up to length scales between mineralized fibrils and lamella, osteons, and entire bone to produce a material that is not only strong and damage tolerant, but also capable of undergoing continuous remodeling. Close to contact collagen-apatite interface also controls the biological mechanisms, such as cell adhesion, mechano transduction, and coordinated resorption and formation by osteoblasts, and this hierarchical architecture is the key template in biomimetic scaffolds design in bone tissue engineering.

No existing protocol is dependable in defeating the problems of large load-bearing bone defects, even in clinical practice where the problem is viewed as

widespread. The gold standard is autografts which unite a combination of osteogenic cells, osteoinductive factors, and an osteoconductive matrix, but they are limited by the volume and strongly affected by the morbidity of the donor site, such as pain, infection, hematoma, risk of fracture, and the durability of the operative period [14, 15]. Allografts are effective but lose cellular activity in processing, decrease in osteoinductivity and are linked to immune reactions, slow incorporation and possible transmission of diseases. Metallic implants and acrylic cements (e.g. titanium hardware, PMMA) provide immediate mechanical stability but are biologically inert and are linked to stress shielding, implant loosening, thermal necrosis and revision surgery. Calcium-phosphate cements and ceramics are osteoconductive and structurally similar to bone, but they are not tough and may be incapable of providing sufficient vascularization and remodeling in large defects. In general, most of the currently available materials lack the hierarchical collagen-apatite architecture and coupled mechanical and biological processes of bone, which drives tissue-engineered, biomimetic strategies, especially the use of collagen-based scaffolds with *in situ* mineralization that is better controlled and thus more reminiscent of the native bone regeneration process [16].

Type I collagen is the main organic component of bone which facilitates the physiologic mineralization process, resulting in the deposition of apatite crystals within a fibrillar skeleton that has intrinsic biocompatibility, low immunogenicity, and natural cell-adhesion sites that can stabilize the adhesion, migration, and differentiation of osteoblasts [17]. Compared to most synthetic polymers, the collagen scaffolds are better able to mimic the structure and breakdown of the native extracellular matrix, which increases their biological compatibility. Nonetheless, plain collagen is not stiff enough and does not have long-term mechanical stability to support any load and this requires the addition of a mineral phase. The *in situ* location and orientation of apatite in and around the collagen fibrils avoids mixing and/or coating, thereby controlling the location and orientation of apatite, and the crystal size, to facilitate intrafibrillar mineralization that closely mimics the hierarchical structure of bone, as previously reported [18, 19]. This biomimetic strategy advances the mechanical properties and biological performance of the *in situ* mineralized collagen scaffolds such as increased osteoconductivity, osteoinductivity and preferential cell-matrix interactions that render *in situ* mineralized collagen scaffolds a promising platform to bone tissue engineering.

This review is devoted to *in situ* mineralization of the collagen-based scaffolds to bone tissue

engineering, highlighting the most important mechanisms and fabrication strategies that control the mineral deposition, structure, and hierarchical organization in the scaffolds. It describes the effect of these structural features on mechanical strength and biological responses including cell adhesion, proliferation and osteogenic differentiation. Other significant translational issues discussed in the review are the manufacturability, reproducibility, sterilization, and regulatory obstacles that need to be met before clinical use. The necessity to focus on standardized processing methods to guarantee the reliable quality and operation of the scaffolds is highlighted. Lastly, it provides future priorities to move these biomimetic scaffolds toward useful applications in bone regeneration therapies, noting the need to more closely couple mechanical competence with the biological activity [20].

Bone Extracellular Matrix as Blueprint Design

Cortical Structure of the bone

The remarkable mechanical and biological performance of bone is due to the high level of hierarchical organization on several scales. The type I collagen fibrils are mineralized at the nanoscale using carbonated hydroxyapatite platelets oriented along the fibril axis, resulting in the formation of mineralized collagen fibrils that give rise to nanomechanical heterogeneities that are important to strength and toughness [6]. At the microscale, these fibrils are arranged in lamellae and osteons in cortical bone or trabeculae in cancellous bone, forming anisotropic architectures that effectively distribute mechanical loads [21]. These building blocks create complex-shaped bones that can continuously undergo remodeling at the macroscale in order to respond to functional needs [6]. Replication of this nano-to-macro hierarchical structure is crucial in the development of biomimetic scaffolds that imparts the same stiffness, toughness, and self-repair capability on bone that is challenging to accomplish using single-phase synthetic materials [22].

This hierarchical view of bone was formalized in the foundational structure mechanical framework of Weiner and Wagner, who described the bone family of materials across the seven discrete levels organization, from individual mineral crystals and mineralized collagen fibrils, through fibril arrays, lamellae and osteons, up to whole-bone architecture, and argued that mechanical competence at each level depends on the specific packing and orientation strategy used at the level below it [23]. This framework remains the reference point against which biomimetic scaffold hierarchies, including those discussed throughout this review, are implicitly compared: a scaffold that reproduces only one or two of these seven levels (typically the nanoscale

mineral-collagen relationship) cannot be assumed to reproduce the mechanical competence of native bone, since emergent toughening mechanisms in natural bone arise from the coupling between adjacent hierarchical levels rather than from any single level in isolation [23].

Collagen Fibril Type I structure

The major structural protein in bone is type I collagen, that is a triple helix made up of two $\alpha 1(I)$ and one $\alpha 2(I)$ polypeptide, bestowing good tensile strength and enzymatic resistance [24]. The molecules self-assemble into fibrils having a typical D-periodic banding pattern with 67 nm gap regions that are preferred for mineral nucleation and overlap regions that are mechanically stable and collectively determine the spatial orientation of the apatite crystals during mineralization [25]. The lateral association of higher order bundles of collagen fibrils gives rise to the anisotropic mechanical properties of bone. The stiffness, toughness and interactions between non-collagenous proteins and collagen fibrils are also a function of their molecular composition, cross-linking density and hydration condition, and thus the structure of the fibrils is an important consideration for bone quality and is a major objective in designing biomimetic scaffolds [26]. Modification of these properties can impart mechanical strength to a collagen scaffold and promote the development of bone tissue engineering applications.

The quarter staggered packing model that underlies this D-periodic banding was first proposed by Hodge and Petruska from electron microscopy of ordered tropocollagen aggregates, and it remains the structural basis on which every subsequent gap-zone nucleation model, including the non-classical pathways discussed later in this review, is built [27]. Three-dimensional reconstructions of mineralized turkey tendon by Traub, Arad and Weiner subsequently confirmed that apatite crystals are not randomly scattered within this gap-zone lattice but

occupy specific, periodically repeating positions with a defined crystallographic relationship to the collagen microfibril, including that the collagen lattice itself imposes a high degree of registration on mineral crystal placement beyond what is provided by local charge alone [28]. This microfibrillar registration is a structural constraint that any biomimetic *in situ* mineralization strategy claiming to achieve “intrafibrillar” mineralization should, in principle, be judged against, rather than against gap-zone infiltration alone.

Intrafibrillar vs Extrafibrillar Mineralization

In bone, mineralization occurs in two major spatial regions, intrafibrillar and extrafibrillar, and both are required to recapitulate native bone properties, as shown in Fig. 1. The intrafibrillar mineralization is the deposition of apatite nanocrystals in the gaps and overlap sites of type I collagen fibrils and determined by the ultrastructure of the fibril and controlled by non-collagenous proteins or their synthetic analogues; this mineral state reinforces collagen and preserves its flexibility, both in terms of transferring nanoscale stress and bone toughness and fatigue resistance [29]. Extrafibrillar mineralization deposits mineral between fibrils and around fibril bundles, enhancing the overall stiffness and compressive strength but may lead to brittleness when preponderant, since weaker organic-inorganic attachment is involved. In native bone, there is a highly organized composition of the two types of mineralization with intrafibrillar mineralization being a characteristic of physiological biomineralization. The objective of biomimetic scaffold design is the creation of such dual mineral distribution, frequently via polymer induced liquid precursor (PILP) to encourage intrafibrillar mineralization and keep suitable extrafibrillar mineral content to achieve a balance between mechanical and biological functionality [30, 31]. These scaffolds have shown better mechanical qualities, biocompatibility, and increased osteogenic potential and can be utilized in bone regeneration.

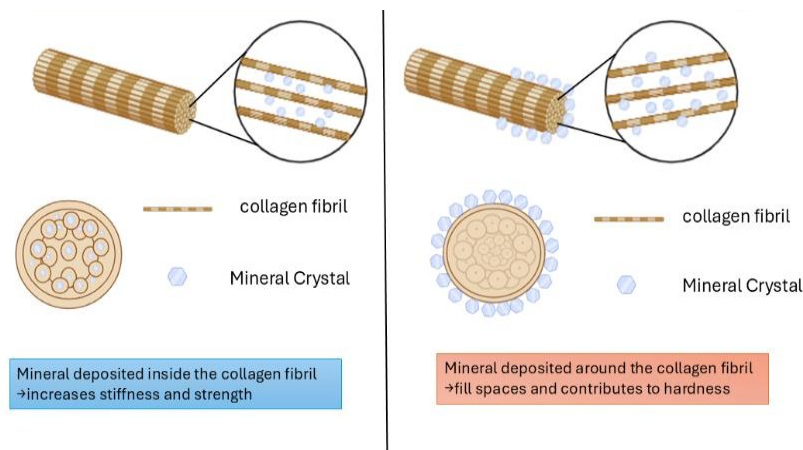


Fig. 1 Intrafibrillar Mineralization vs Extrafibrillar Mineralization

Non-collagenous protein role and biomimetic analogue role

Non-collagenous protein (NCPs) of bone, such as osteopontin, bone sialoprotein, dentin matrix protein-1, and Sibling family members, are key regulators of mineralization although they are lowly expressed [18, 32]. These proteins contain acidic amino acids and phosphorylated residues which allow them to bind strongly to calcium and phosphate ions to regulate nucleation, crystal growth, orientation as well as spatial confinement of apatite in collagen fibrils, and consequently, intrafibrillar mineralization, a major aspect of physiological bone formation [33]. Amorphous calcium phosphate precursors are also stabilized by NCPs and direct their infiltration into collagen gap regions, which connects mineralization chemistry to biological processes including cell-matrix interactions, osteoclast resorption and remodeling. The difficulty in isolating native NCPs (naturally) to use in clinical practice has led to the production of synthetic biomimetic analogues such as poly (aspartic acid), poly(acrylic acid), phosphorylated polymers, polyphosphates, and casein phosphopeptides to mimic NCP activity by stabilizing amorphous precursors and guiding mineral infiltration through the polymer-induced liquid precursor (PILP) mechanism [34, 35]. The analogues allow regulated intrafibrillar and extrafibrillar mineral deposition that improves the nanoarchitecture, mechanical properties, and biological functionality of collagen scaffolds in bone tissue engineering. The rational design of NCP-based mineralization is thus a key challenge to the development of new generation *in situ* mineralized collagen scaffolds that are highly reminiscent of the native bone extracellular matrix [32].

It should also be recognized that the regulatory template of native bone extends beyond the classical acidic NCPs to the proteoglycans (PGs) of the extracellular matrix and their glycosaminoglycan (GAG) side chains. In particular, the small leucine-rich proteoglycan decorin and biglycan, together with the extent and type of their post-translational sulfation and GAG-chain modification, regulate collagen fibrillogenesis, intrafibrillar spacing, matrix hydration and mineral deposition [36]. Biglycan-deficient and decorin-deficient animal models show altered collagen fibril diameter, reduced bound-water content and compromised fracture toughness, and biglycan in particular has been shown to exert a predominant, largely non-redundant influence on trabecular bone mass and toughness that decorin does not fully compensate [36] because proteoglycans and their GAG chains,

and not only the acidic NCPs, form part of the biological template that *in situ* mineralization strategies aim to emulate, incorporating proteoglycan or GAG mimetic components alongside NCP-analogue polyelectrolytes is an underexplored design axis for biomimetic collagen scaffolds that merits further investigation.

***In situ* Mineralization Scaffolds that are based on collagen**

Sources of collagen

Scaffold fabrication collagen is obtained from many biological sources, each with its own advantages and disadvantages for application in *in situ* mineralization. Bovine collagen is popular because of its accessibility, well-characterized mechanical properties, and affordability, but presents risks of immunogenicity, disease transmission, and cultural limitations [37, 38]. Porcine collagen is similar to human collagen in biochemical and mechanical properties, including cell adhesion and mineral deposition, but has similar immunological and ethical issues as bovine collagen, and also has batch variability [38]. Collagen obtained by extracting fish skin, scales, or jellyfish is a marine-derived collagen with benefits including a reduced zoonotic risk, reduced cultural barrier, and excellent biocompatibility, but with reduced thermal stability and mechanical strength, which can be enhanced through crosslinking or blending [39-41].

Human or recombinant collagen offers the best biocompatibility and ability to control molecular properties accurately, ensuring the absence of species-specific immune response, yet it costs a lot to produce and cannot be scaled widely to a large clinical utilization at the present. In general, the collagen source influences the stability of the scaffold, potential to mineralize, and transplantability, and current studies aim to optimize marine and recombinant collagens to be utilized in bone tissue engineering in the future [39, 40, 42].

Forms of scaffolds

The scaffolds made of collagen can undergo *in situ* mineralization in many different structural forms and each has distinct advantages in regulating mineral deposition, mechanical characteristics, and biological activity. Hydrogels offer a highly hydrated, three-dimensional environment, resembling native extracellular matrix, that allows minerals to be uniformly mineralized using ion diffusion, but typically needs crosslinking or mineral reinforcement to overcome their low mechanical strength [43, 44]. Sponges are commonly fabricated via freeze-drying and are

designed with interconnected macroporosity enabling cell penetration, vascularization and nutrient delivery, which allows mineral deposition throughout the scaffold, but obtaining uniform distribution of minerals in thicker sponges has proven difficult [20]. In guided bone regeneration, membranes act as barriers to surface mineralization and allow controlled surface mineralization, but restrain cell penetration and integration compared to 3D constructs [20].

The high-level 3D-printed scaffolds have offered the control of geometry, pore size and spatial patterns of mineralization, which have resulted in hierarchical and patient-specific scaffolds, but collagen integrity during printing and reproducible mineralization on scale are still issues [45]. New methods like cryogenic 3D printing and 4D printing are already improving scaffold biomimicry and functionality by facilitating complex architecture and dynamic shape change to irregular bone defects. All these scaffold shapes in general offer a flexible framework to design mineralized collagen scaffolds to suit clinical requirements in bone tissue engineering [20, 43].

Crosslinking strategies and their effect on mineralization

Crosslinking plays an important role in the stabilization of collagen-based scaffolds and also plays a significant role in *in situ* mineralization by altering the packing of fibrils, charge distribution and ion movement within the matrix. Physical crosslinking techniques, including dehydrothermal treatment, ultraviolet irradiation, and ionic interactions, improve the stability of scaffolds without adding chemicals, retain collagen bioactivity, and allow intrafibrillar mineralization with no mechanical reinforcement since they efficiently diffuse ions, but form weaker reversible crosslinks [46]. Strong covalent bonds are formed by chemical crosslinkers such as glutaraldehyde, carbodiimide (EDC/NHS) and genipin, which enhance mechanical strength and enzyme resistance, but the creation of too many crosslinking types may decrease fibril porosity and obscure charged groups, inhibiting intrafibrillar mineral nucleation and promoting extrafibrillar deposition, as well as raising cytotoxicity and crosslinker residue [47].

Enzymatic crosslinking, with transglutaminase or lysyl oxidase mimetics, is a biomimetic strategy that preserves the native structure and bioactivity of collagen and provides sufficient stability, allowing a more uniform distribution of minerals and increased cell compatibility [48]. Optimize the density of the crosslinking and ensure that there is a good balance of mechanical stability with mineralization capacity since a very dense network might prevent the formation of intrafibrillar minerals and a too low density would cause loss in the scaffold's

mechanical stability. Based on the recent reports, collagen-hydroxyapatite composite with enhanced mechanical properties and osteogenic potential as a bone tissue engineering tool could be achieved by utilizing proper crosslinking methods and biomimetic mineralization techniques [46].

***In Situ* Mineralization in Collagen Scaffolds Mechanisms**

In classical theory of nucleation and growth, the nucleus forms and develops within the supersaturated solution. Classical nucleation and growth theory describes mineral formation as a thermodynamically regulated process in which ions present in a supersaturated solution overcome an energy barrier to generate stable nuclei, which is then followed by crystal growth by the attachment of ions [49]. This process is controlled by local calcium and phosphate ion supersaturation, pH, and temperature as well as charged functional groups on collagen fibrils in collagen-based scaffolds.

Equally critical to supersaturation, though less often discussed, is the stoichiometry of the available calcium and phosphate ions and the relative proportion of the phosphate species in solution PO_4^{3-} , HPO_4^{2-} , H_2PO_4^- . Since these govern which calcium phosphate phase (e.g., dicalcium phosphate dihydrate, octa calcium phosphate, defect or carbonated apatite) nucleates preferentially and how closely the resulting mineral approaches the Ca/P molar ratio of ~1.5-1.67 that characterizes biological apatite [50]. Because the fractional abundance of HPO_4^{2-} versus PO_4^{3-} is itself strongly pH dependent, Ca/P stoichiometry and phosphate speciation cannot be treated as independent of the pH effects discussed later in this review; both must be reported and controlled together for mineralization outcomes to be reproducible and comparable across studies [51].

The negative charges of the residues, particularly the carboxylate groups, concentrate the calcium ions and reduce the nucleation energy barrier causing heterogeneous nucleation at the surface of the fibrils and resulting in extrafibrillar mineralization with comparatively large and well-crystallized apatite crystals [52, 53]. Nevertheless, in classical nucleation, mineral growth tends to be extracellular to nucleation sites and so does not penetrate the interior of collagen fibrils and so does not recapitulate *in vivo* nanoscale intrafibrillar mineral architecture of native bone.

Experiments reveal that confined collagen geometry in fibrils lowers nucleation energy barriers, promoting intrafibrillar mineralization by modifying nucleation pathways and mineral morphology, which cannot be completely attributed to classical nucleation [52, 53]. As such, although classical nucleation can be used to increase scaffold stiffness

and surface bioactivity by means of extrafibrillar mineralization, further mechanisms or biomimetic approaches are required to create the hierarchical intrafibrillar mineralization that is essential to bone-like functionality.

Non-classical pathways (PILP, cluster aggregation, Liquid-phase precursors)

These non-classical mineralization pathways play a key role in the intrafibrillar mineralization of collagen-based scaffolds since they facilitate the mineral infiltration of the confined collagen fibril spaces, which cannot be entirely accounted for by classical ion-by-ion growth. One of the main mechanisms is the polymer induced liquid precursor (PILP) process in which amorphous calcium phosphate (ACP) is stabilized in a liquid-like form by acidic polymers or non-collagenous protein analogues to enable the infiltration of collagen gap regions by capillary forces and electrostatic forces and subsequent conversion to oriented apatite nanocrystals within fibrils [54]. Cluster aggregation mechanisms postulate that prenucleation ion clusters can act as stable building blocks that can reorganize into crystalline apatite without classical nucleation barriers, allowing spatial control of mineral formation within the collagen matrix [55].

At this point it is important to be explicit about what is meant by “amorphous calcium phosphate” in this review, since the term is used in the literature to describe two distinct states that are frequently conflated. The first is a morphologically amorphous, liquid-like or gel-like precursor droplet with no defined particle shape; the second is a chemically/crystallographically amorphous phase that genuinely lacks long-range atomic order, as verified by electron or X-ray diffraction. Chemically amorphous ACP is thermodynamically metastable at physiological pH and ionic strength and transforms into poorly crystalline apatite within seconds unless its surface is deliberately “crystal-poisoned” by stabilizing additives such as Mg^{2+} , citrate, pyrophosphate, or acidic NCP/polymer analogues; genuinely amorphous ACP is stable in solution for extended periods only at pH values of approximately 9 and above [49, 56]. A consequence of this, and a constraint that *in situ* mineralization protocols must confront explicitly, is the same additives used to prolong the liquid-precursor stage so that it can infiltrate the collagen gap zones also inhibit its subsequent crystallization; conversion to oriented apatite therefore requires that the stabilizing additive be at least partly displaced, diluted or degraded once the precursor has infiltrated the fibril, and protocol that do not address this removal step risk trapping the precursor in a permanently disordered state rather than achieving the intended intrafibrillar

apatite [54].

Pathways to liquid-phase precursors are directed towards hydration of amorphous intermediates and their high mobility, allowing highly mobile intermediates to access nanoscale compartments inaccessible to crystalline phases, and forming biomimetic mineral assemblies that can be compared with naturally occurring bone [57]. Current experimental data suggest that the rate and mechanical properties of intrafibrillar mineralization can be controlled through the concentration of polymer: low concentrations of polymer favor fast mineralization, while high concentrations of polymer favor high strength. All of these non-classical pathways provide mechanisms for the formation of bone-like minerals and a starting point for future biomimetic mineralized collagen scaffold manufacturing to improve osteogenic potential and hierarchy.

Historical controversy: is amorphous calcium phosphate a genuine *in vivo* precursor?

It is worth making explicit, rather than assuming settled, that the ACP-precursor model underpinning most of the non-classical pathways above rests on a long-running and not fully resolved controversy within the biomineralization field, which this review’s discussion of collagen scaffolds inherits directly. Early proponents of a mineral precursor in bone framed the question mainly in terms of the composition and crystallinity of the first formed mineral phase itself [9, 49]. Decades later, high-resolution cryo-electron and scanning electron microscopy of the continuously growing fin bony rays of zebrafish, used precisely because mineralization there is spatially and temporally resolved along a single growing element, showed that newly deposited mineral is present as abundant, genuinely disordered amorphous calcium phosphate particles that crystalline progressively as the tissue matures, providing some of the strongest direct evidence yet for a transient ACP precursor operating *in vivo*, not only *in vitro* [58, 59]. This Finding reinvigorated the case for PILP-type mechanisms as biomimetic rather than purely synthetic constructs. At the same time, structural studies of mature, already-mineralized collagen fibrils by three dimensional reconstruction methods describe apatite crystals occupying highly ordered, periodically repeating positions within the collagen lattice, a degree of order that is not, on its own, proof of how the mineral first arrived and does not by itself distinguish between a transient amorphous-precursor pathway and more direct classical nucleation pathways operating alongside it [13, 28]. The two bodies of evidence are not strictly contradictory, since a transient amorphous phase could still crystalline into a highly ordered final

lattice, but they do reflect two different emphases in the field: one tradition (Weiner, Addadi, Mahamid and co-workers) that foregrounds the amorphous precursor state and its transformation kinetics, and another (Glimcher, Landis and co-workers) that foregrounds the final crystalline architecture and its relationship to collagen ultrastructure [9, 13, 28, 58, 59]. For the purpose of *in situ* mineralization of collagen scaffolds, this matters practically rather than merely academically: PILP-type protocols are engineered on the explicit assumption that a genuinely amorphous, liquid-like precursor phase is both real and necessary for intrafibrillar mineral placement, and the zebrafish-fin evidence is the closest direct *in vivo* support that assumption currently has, but the field has not yet established how universally this pathway operates across bone types, species, and development stages, and scaffold designers should treat the ACP-precursor mechanism as a well-supported working model rather than an established universal law of bone mineralization.

Polymer-induced liquid-precursor (PILP) systems role

A liquid-precursor (PILP) system based on polymer stabilization of amorphous calcium phosphate (ACP) in a liquid-like form by acidic polymers like poly(aspartic acid) or poly(acrylic acid) is a successful attempt to recreate native bone mineralization. This precursor is a metastable state which enters the collagen fibril gap spaces through capillary and electrostatic interactions and transforms into oriented apatite nanocrystals aligned along the collagen axis where the location, size and orientation of these apatite crystal orientations are tightly controlled as found in native bone nanostructure [31]. Mineralization with PILP can enhance mechanical properties of scaffolds, including stiffness and toughness, while maintaining the flexibility of the collagen, and may be controlled by the type of polymer, its molecular weight, concentration, and mineralization conditions to tune mineral content and distribution. There are challenges to clinical use of PILP systems: sensitivity to processing parameters, scalability, and biocompatibility and regulatory acceptance of polymer additives.

Crosslinking in combination with PILP mineralization can be employed to further improve the mechanical properties, but with the mineral content having to be optimized to prevent detrimental effects on tensile properties. Overall, PILP has the potential to be a new promising biomimetic method for the fabrication of mineralized collagen scaffolds with bone-like hierarchical structure and osteogenic properties, although some challenges remain to be addressed

toward its practical implementation.

Ion-mediated mineralization systems

Controlled delivery of bioactive ions in collagen scaffolds: The main ion-mediated mineralization approaches to induce apatite formation provide tunable and relatively easy *in situ* mineralization pathways which can also be used to tune the mineral phase to control biological responses. Applicable calcium phosphate (CaP) systems are known to recapitulate bone mineral composition by allowing apatite to precipitate by heterogeneous nucleation by varying Ca/P ratios and solution conditions, but the preponderance of mineral in extrafibrillar forms is frequent unless supplemented with non-classical approaches such as PILP [56].

Amorphous calcium phosphate is stabilized, excessive crystal growth inhibited, and finer, less crystalline apatite is produced by magnesium doping, enhancing the mineral homogeneity and osteogenic and angiogenic response, although high Mg concentrations can inhibit mineralization; citrate can overcome Mg inhibition effects by altering collagen electronegativity and hydration layers [60]. The apatite scaffold Sr substitution stimulates osteoblasts and inhibits osteoclast resorption, increasing bone formation *in vivo* with sustained Sr release correlating with osteo-anabolic effects, but needs to be controlled regarding release kinetics to prevent systemic side effects.

This safety margin deserves particular emphasis: above certain local concentrations, Sr²⁺ can act as a mild calcium ion channel blocker and can compete with Ca²⁺ at calcium-sensing receptors and calcium-dependent signaling pathways used by osteoblasts and osteoblasts themselves. Scaffold Sr loading and release osteoblast stimulation and osteoblast inhibition without inhibiting the calcium channel mediated signaling on which normal bone cell function depends; this concern is distinct from, and additional to, the risk of systemic side effects associated with sustained Sr release noted above.

Silicon-based systems stimulate early mineralization, stimulate collagen production, osteogenic differentiation and mineral-collagen interfacial bonding; increasing mineralization kinetics without compromising silicate stability is difficult [61].

pH and ionic strength effects, supersaturation

The physicochemical environment plays a vital role in *in situ* mineralization in collagen scaffolds by controlling the kinetics of mineral nucleation, phase stability and spatial distribution.

pH affects calcium-phosphate speciation and collagen charge. Chemically/crystallographically amorphous calcium phosphate is only stable for

extended periods at alkaline pH of approximately 9 and above; at near-neutral to mildly acidic pH which is typical of the physiological and most *in situ* mineralization conditions used in the field, ACP is short-lived and transforms rapidly toward more soluble transient phases (such as dicalcium phosphate dihydrate) or directly toward poorly crystalline apatite unless a crystal-poisoning stabilizer is present [49, 56]. In parallel, pH governs the relative proportions of PO_4^{3-} , HPO_4^{2-} and H_2PO_4^- , and hence the effect Ca/P stoichiometry available for mineral formation: near-neutral pH favours HPO_4^{2-} as the dominant phosphate species and yields non-stoichiometric, calcium-deficient apatite, where alkaline pH shifts the equilibrium toward PO_4^{3-} and more stoichiometric hydroxyapatite [50, 51]. pH also modulates collagen's carboxylate ionization, impacting calcium binding and nucleation efficiency.

The ionic strength regulates the electrostatic interactions between ions, collagen, and mineral precursors; high ionic strength has the ability to screen electrostatic interactions, facilitating bulk precipitation, and low ionic strength facilitates precursor penetration into collagen fibrils, allowing more precise mineral deposition to occur [62].

Mineral formation depends on supersaturation, which increases nucleation rate with increasing supersaturation, but tends to result in extrafibrillar precipitation and poor structural control in high supersaturation, and in interfibrillar mineralization and metastable amorphous precursor formation in moderate supersaturation, which better recapitulates bone architecture [62]. Mineral phase and crystallinity are also dependent upon the scaffold microstructure and temperature, with stiff collagen matrices favoring stoichiometric hydroxyapatite formation and elevated temperatures enhancing crystallinity and calcium saturation. Optimization of these parameters increases the mechanical properties and osteogenic potential of scaffolds, where hierarchical, biomimetic mineralization of collagen scaffolds has been demonstrated through molecular control of pH, ionic strength and supersaturation to enhance bone tissue engineering [62].

Surface charge (Zeta-potential) effects

The surface, or zeta, potential of collagen fibrils, non-mineralized scaffolds and mineralized scaffolds is a further physicochemical variable that governs *in situ* mineralization but is rarely reported in the collagen-scaffold literature. The net surface charge of collagen is normally electronegative at physiological pH owing to exposed carboxylate residues, but the molecule also carries a localized, relatively electropositive patch near its C-terminal telopeptide region; the interplay of these domains

determines the initial electrostatic attraction and subsequent infiltration of calcium ions and anionic mineralization-directing macromolecules into the fibril, and hence the balance between intrafibrillar and extrafibrillar nucleation [19, 63]. Surface-modification strategies, such as silica or polyelectrolyte functionalization of collagen, measurably shift the isoelectric point and zeta-potential of the substrate and correspondingly alter apatite nucleation density and bioactivity in simulated body fluid. Beyond its role in mineral conformation and integrin engagement. Systematically characterizing and reporting the zeta-potential of collagen scaffold before, during and after mineralization would therefore allow better correlation between mineral distribution and downstream cellular responses; its omission from most reports in this field currently limits comparison between studies and is identified here as a gap for future studies work.

Kinetics of intrafibrillar vs extrafibrillar mineral deposition

The speed and order of mineral deposition have a vital effect on the formation of minerals within collagen fibrils (intrafibrillar) or outside collagen fibrils (extrafibrillar). Intrafibrillar mineralization is a slow, kinetically limited process which involves regulated nucleation and growth of metastable precursors, including amorphous calcium phosphate, which may infiltrate the small zones between collagen in the collagen gap; this process is preferred by low-to-moderate supersaturation and stabilized precursor phases, permitting slow mineral infiltration and robust collagen reinforcement [64]. On the contrary, the extrafibrillar mineralization occurs at very high rate, reaching high supersaturation, which causes the rapid nucleation and crystal growth outside the fibrils, and after a short period the mineral is more content and stiff, but, if the supersaturation is too high, the toughness is reduced. Timing of these processes is controlled by native bone mineralization, where early intrafibrillar mineralization creates a reinforced collagen template and an extrafibrillar growth which reinforces bone [65].

Polymer additives, like polyacrylic acid and glycosaminoglycans, can also influence the kinetics of mineralization and/or the mineral polymorphs, and thus the ratio between intra- and extrafibrillar mineral deposition. This kinetic balance *in vitro* has been difficult to achieve, but is necessary to reproduce bone-like hierarchical structures and mechanical properties in collagen scaffolds. The rate of mineralization, the stability of precursors and diffusion are factors which can be understood and controlled to provide biomimetic mineralized collagen materials with improved mechanical

properties [66].

Advanced and real-time characterization tools to examine mineralization

To characterize *in situ* mineralization processes in collagen scaffolds, methods that measure structural and chemical changes across scales and time are needed. TEM and high-resolution TEM (which are frequently used together with selected area electron diffraction, SAED) are used to visualize intrafibrillar and extrafibrillar mineral deposition, crystal orientation, and crystal crystallinity at the nanoscale in collagen fibrils [67]. Small and wide-angle X-ray scattering (SAXS/WAXS) is a quantitative and statistically robust method for measuring particle size, orientation and hierarchical structure of minerals, which distinguishes amorphous mineral precursors from crystalline phases of the development of mineralization [31]. Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy are used to trace the changes in chemical composition, mineral-to-matrix ratio, and

phase change between amorphous calcium phosphate and apatite and can be used to gain insight into mineral maturation [68].

The use of advanced techniques such as cryo-electron microscopy (cryo-EM) and *in situ* liquid-cell TEM makes it possible to observe transient liquid or amorphous precursors phases directly, which support non-classical mineralization pathways. Atomic force microscopy (AFM) is an extension of these and measures nanoscale mechanical characteristics to correlate the distribution of minerals with the local stiffness and deformation behavior and is essential in understanding the mechanics of scaffolds. These complementary methods, when used together, form powerful mechanism-structure interactions that are necessary in designing biomimetic mineralized collagen scaffold that closely resembles native bone properties. Fig. 2 shows the mechanisms of *in-situ* mineralization in collagen scaffolds.

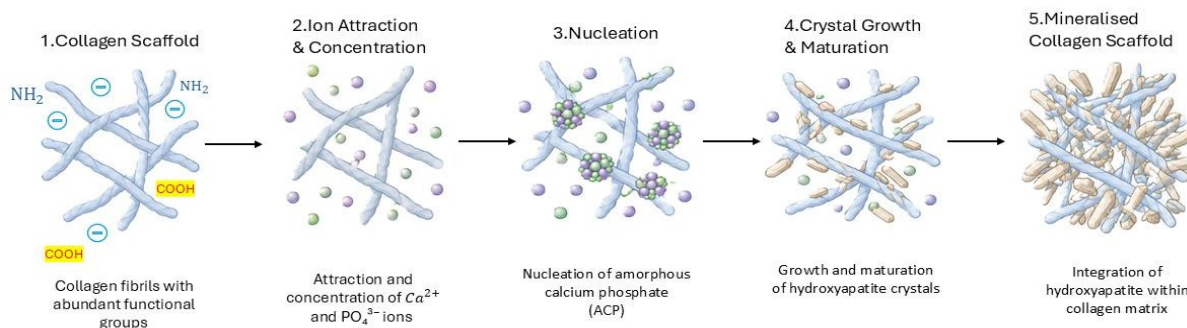


Fig. 2 Mechanisms of *In situ* Mineralization in Collagen Scaffolds

In situ mineralization strategies

Non-collagenous protein (NCP) analogues biomimetic mineralization

The biomimetic mineralization strategies aim to recapitulate the regulatory roles of natural non-collagenous proteins (NCPs) in bone mineralization with the aid of synthetic or biologically inspired analogues. Acidic and phosphorylated NCPs in natural bone control the nucleation of calcium phosphate, stabilization of amorphous precursors, and orientation of mineral into collagen fibrils. Poly(aspartic acid), poly(acrylic acid), poly(glutamic acid) and phosphorylated polymers have been extensively used to mimic these functions in collagen scaffolds [69, 70]. These analogs of NCP bind calcium ions strongly, reducing interfacial energy, stabilizing amorphous calcium phosphate (ACP) precursors, which can proceed to intrafibrillar mineralization with better control of the size, distribution, and orientation of the mineral than

with the standard method of calcium phosphate precipitation [70].

The effectiveness of such biomimetic mineralization is determined by the polymer chemistry, molecular weight, concentration, and affinity of collagen binding; too much polymer may prevent crystallization or weaken mechanical properties, whereas too little may result in uncontrollable extrafibrillar mineral deposition. Although they have proven useful as research tools, issues like polymer residues, batch variability, and regulatory issues hamper their clinical translation, and thus, there is a need to ensure that these systems are optimized and validated carefully [71].

Stimulated body fluid based *in situ* mineralization

Another early and popular approach to mineralizing collagen scaffolds is simulated body fluid (SBF) based mineralization, which attempts to recreate the

ionic concentration of human blood plasma. The process of mineralization takes place due to the spontaneous calcium-phosphate precipitation when the scaffolds are left in the SBF over long periods, and when the collagen fibrils contain negatively charged groups that act as the nucleation centers through which apatite can be deposited [72]. This method has been preferred due to its simplicity, low cost and physiological relevance and augmentation of scaffold bioactivity through augmentation of osteoconductivity, cell adhesion, and early osteogenic differentiation. Nevertheless, mineral deposition through SBF usually proceeds according to classical nucleation routes and leads to extrafibrillar mineralization with comparatively large and poorly regulated crystals, whereas intrafibrillar mineralization is difficult since surface precipitation is rapid and the precursor is not readily infiltrated [72]. Several adjustments have been investigated to enhance mineral control (including ion concentration, alternate soaking cycles, the presence of magnesium or carbonate ions, and pre-functionalization of collagen with charged molecules) to make SBF methods more like the nanoscale collagen-apatite architecture of native bone. SBF-based mineralization is thus most appropriate as a screening methodology of surface bioactivity, as opposed to an entirely biomimetic approach to hierarchical bone regeneration [73].

The use of SBF in this field traces back to the protocol formalized by Kokubo and Takadama, who proposed that a material's capacity to nucleate bone-like apatite on its surface when soaked in an acellular fluid approximating the ion concentrations of human blood plasma could be used as a practical, animal-sparing predictor of *in vivo* bone-bonding ability, and who catalogued a substantial correlation between *in vitro* apatite formation and *in vivo* bioactivity across a range of established bioceramics [74]. However, the method's biological realism has itself been directly challenged in the literature: Bohner and Lemaître argued that conventional SBF is a poor mimic of the buffering systems present *in vivo*, is typically prepared at supraphysiological ionic supersaturation relative to true extracellular fluid, and has produced documented false positives and false negatives when a material's *in vitro* apatite-forming behavior was compared against its actual *in vivo* bone-bonding outcome [75]. For collagen scaffold studies specifically, this critique is directly relevant: because SBF immersion is the cheapest and most widely used *in situ* mineralization screen in the literature reviewed in this article, results reported as "Bioactive" or "Osteoconductive" on the strength of SBF mineral deposition alone should be read as a preliminary surface-bioactivity signal rather than as evidence of the scaffold's eventual *in vivo* performance, a

distinction this review's earlier characterization of SBF as a screening tool [72, 73] is intended to reflect but which is worth stating plainly given how often the two are conflated in the primary literature.

Enzyme-mediated mineralization

Mineralization strategies based on enzymes involve the use of biological catalysts to produce mineral-forming ions in a spatially and temporally regulated way, akin to physiological bone formation. This process involves alkaline phosphatase (ALP) that dissolves organic phosphate esters to inorganic phosphate, enhancing local supersaturation and facilitating intrafibrillar mineral deposition with stabilized amorphous precursors or NCP analogues with or without ALP incorporated into scaffolds [76].

Another alternative is urease-mediated mineralization that increases local pH and carbonate concentration through urea hydrolysis but which typically results in extrafibrillar mineralization and increases cytocompatibility issues because of ammonia byproducts, which constrain its application in practice [77]. Enzyme-mediated methods have been shown to offer biologically inspired control over mineralization kinetics and localization despite challenges like enzyme stability, activity loss during processing, and cost, and future clinical translation of enzyme-mediated methods relies on improved enzyme stability, scalability, and complementation with other biomimetic methods. More recent developments are enzyme-activated biomimetic vesicles, and enzyme-responsive hydrogels which extend the capability of spatial and temporal control of mineralization to enhance osteogenic differentiation and bone regeneration outcomes.

Ion-chelation and controlled-release-driven mineralization

Controlled-release and ion-chelation approaches control *in situ* mineralization by adjusting the local concentration of calcium and phosphate ions in the collagen scaffolds to avoid bulk precipitation, and allow local control of mineral deposition. The removal of calcium ions through the temporary binding of citrate, EDTA, or naturally occurring polycarboxylates reduces the supersaturation and postpones nucleation, which enhances the gradual release of ions and a more uniform mineral formation in the scaffolds [78]. Microspheres, nanoparticles or layered-reservoir controlled-release systems release calcium, phosphate or bioactive ions over longer durations, promoting sustained mineral growth and enhanced distribution of minerals through thick scaffolds.

These methods minimize uncontrolled extrafibrillar precipitation and improve the homogeneity of the

mineral, but too much chelation can suppress mineralization and inadequate release systems can lead to the heterogeneous deposition of the mineral, compared to direct ion immersion [79]. The biocompatibility and regulatory approval of chelators and carriers are also still major concerns to clinical translation. Optimized, the ion-chelation and controlled-release techniques provide highly adaptable platforms to precisely tune mineralization dynamics and incorporate mineral formation with scaffold degradation and tissue regeneration.

Stimuli-assisted mineralization

The idea of stimuli-assisted mineralization strategies is based on external physical stimuli like electric, magnetic and mechanical stimulation to control the ion transport, nucleation and crystal growth of collagen scaffolds, based on the active physical environment of native bone as shown in Table 1. High electric fields cause rapid precipitation which will damage the scaffold; however, electric fields

can promote directionality of ion migration and growth of minerals, and thereby enhancing the alignment of mineral along collagen fibrils [80]. Magnetic field-assisted mineralization involves the spatial control of mineral nucleation by adding magnetically responsive additives, and has been limited to collagen scaffolds and wondered about regarding biocompatibility of nanoparticles and long-term effects.

Mechanical stimulation techniques like cyclic compression and dynamic perfusion increase the ion transport and physiological loading and lead to uniform mineral deposition, in addition to its other effects, while at the same time introducing more complexity and scaling challenges [81]. The latter ones are piezocatalytically-induced mineralization with piezoelectric polymers, and physical stimulation of osteogenic differentiation for bone regeneration, showing promising results.

Table. 1 *In Situ* Mineralization Methods vs Outcomes

Method	Core mechanism	Typical mineral phase	Intra vs Extra Fibrillar	Control Level	Key Advantages	Key Limitations	References
SBF Immersion	Supersaturation driven surface nucleation	Carbonated Apatite	Mostly Extrafibrillar	Low	Simple, Low Cost	Poor nano control, surface dominant	[72]
PILP Process	Polymer stabilized liquid amorphous ACP precursor	Apatite from ACP precursor	Intra + Extra	High	Best biomimetic hierarchy	Parameter sensitive	[16, 56]
NCP Analogue	Acidic polymer/protein mimics stabilize ACP	Nano apatite	Predominantly Intrafibrillar	High	Mimics natural control proteins	Additive safety concerns	[69]
ALP Enzyme	Enzymatic phosphate release → local supersaturation	Apatite	Mixed	Medium	Biologically relevant trigger	Enzyme instability, cost	[76]
Ion Chelation	Gradual ion availability via chelators/carriers	Tunable Ca-P phases	Mixed	Medium-High	Better spatial uniformity	Over-chelation inhibits mineralization	[78]
Ion Doped Systems	Substituted ions growth (Mg/Sr/Si)	Doped apatite	Mostly extra + partial intra	Medium	Adds bioactivity signals	Precise composition control needed	[79]
Stimuli Assisted	Electrical/mechanical fields alter kinetics	Ca-P apatite	Mixed	Experimental	Directional control possible	Low reproducibility	[80]

Structure-Function Relationships in *In situ* Mineralized Collagen Scaffolds

Functional performance of *in situ* mineralized collagen scaffolds is determined by the availability of mineral as well as space distribution, structure and dynamic incorporation of the collagen matrix as

shown in Table 2. This fine hierarchical structure is what provides native bone with its special properties of stiffness, toughness and biological activity. Therefore, understanding the relationship between scaffold structure and performance is crucial to the ability to apply biomimetic mineralization techniques to develop clinically relevant bone replacement scaffolds. This fusion has impacts on mechanical properties, osteogenic potential and

cellular responses essential to bone regeneration. Designed scaffolds with optimized structure-function relations can be more like natural bone, leading to improved tissue repair outcomes. Conversely, the particular information about mineral phases/collagen interactions on different scales is used to develop better scaffolds to be applied in the scope of regenerative medicine.

Table. 2 Structure & Function Relationship Evidence

Structural Feature	Mechanical Effect	Biological Effect	Evidence Type	Reference
Intrafibrillar Mineral	Toughness ↑, Fatigue resistance ↑	↑ Osteogenic differentiation	TEM + Mechanical + cell studies	[6, 29, 82]
Extrafibrillar Mineral	Stiffness ↑, Ductility ↓ if excessive	Osteoconductive surface	Mechanical + In vitro	[29]
Nano sized Apatite	Better stress transfer	↑ MSC adhesion & ALP activity	In Vitro	[52, 53]
Oriented Crystals	Anisotropic strength	Guides cell alignment	Cell assays	[18, 19]
Hierarchical Distribution	Balanced modulus + toughness	Better tissue integration	Multi-scale studies	[73, 82]
Over Mineralization	Brittleness	Reduced cell infiltration	Mechanical + In vivo	[83]

Location and hierarchy of minerals versus mechanical performance

The mechanical behavior of mineralized scaffolds is extremely dependent on the location of mineral relative to collagen fibrils as shown in Fig. 3. The intrafibrillar mineralization, in which apatite nanocrystals are generated in the gap and overlap areas between collagen fibrils, makes the collagen matrix much stronger without reducing its capacity to dissipate energy, allowing the collagen to transfer stress efficiently and increase toughness and fatigue resistance like native bone [82]. Extrafibrillar mineralization deposits, in contrast, place minerals

on scaffold surfaces or between fibers, making them rigid, but frequently brittle, because of the lack of mechanical integration with collagen. It has been demonstrated repeatedly that scaffolds that contain more intrafibrillar mineralization perform better than extrafibrillar or surface mineralization scaffolds in mechanical performance, despite similar total mineral content. This underscores the fact that the hierarchical arrangement of the mineral in the collagen matrix is more crucial as compared to total mineral loading in load-bearing applications. Maximization of intrafibrillar mineralization, therefore, is a promising path towards the creation of biomimetic scaffolds with enhanced mechanical strength and biological capacity [84].

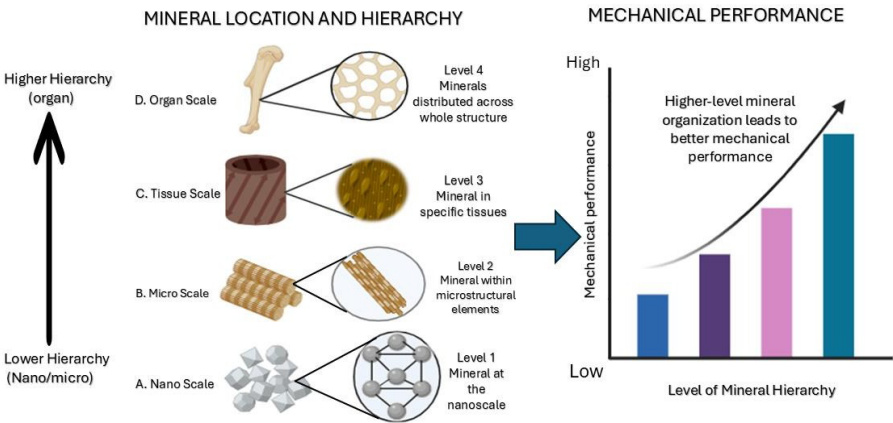


Fig. 3 Mineral Location and Hierarchy vs Mechanical Performance

Nanoarchitecture of minerals and bioreaction

The mechanical properties are not the sole important role of the nanoarchitecture of the mineral phase in controlling cellular behavior. Mineral crystal size, orientation, and surface chemistry have been found to affect protein adsorption, integrin-mediated cell adhesion, and osteogenic signaling, and biomimetic mineralized collagen scaffolds have been found to promote better osteoblast attachment, proliferation, and differentiation than scaffolds with randomly distributed or oversized minerals [84]. Immune responses are also regulated by mineral nanoarchitecture that is essential in bone regeneration; biomimetic scaffolds have the ability to polarize macrophages to a pro-healing M2 phenotype, which suppresses inflammation and promotes angiogenesis to facilitate a conducive environment to bone formation and remodeling [85]. Regulated intrafibrillar and finely dispersed extrafibrillar mineral phases aid in the suppression of inflammatory cues and the promotion of vascularization, which underscores the significance of immunomodulation and osteogenesis [86].

Pattern of mineralization vs degradation and remodeling

The mineralization trend has a strong impact on the rates of scaffold degradation, as well as on the long-term remodeling results. Mineralization within the fibrils holds the collagen fibrils together to prevent rapid breakdown by enzymes but allows gradual breakdown and replacement by new bone, correlating scaffold degradation with tissue regeneration. High extrafibrillar mineral density scaffolds, on the other hand, experience slow or stunted remodeling due to a low level of cell and enzyme accessibility, which may prevent vascular infiltration and unabsorbed residual mineral density that may interfere with integration [87]. The remodeling capacity of native bone is also achieved by the complex and difficult-to-reproduce hierarchically balanced mineral distribution. These findings highlight the necessity of *in situ* mineralization strategies based on mineral deposition patterns in relation to the physiology of degradation and remodeling rather than on mineralization rates. This will play an important role in developing scaffolds for both mechanical and biological integration in the long-term.

Biological Performance of *In Situ* Mineralized Collagen Scaffolds

The integration of mineralized collagen scaffold into the biological environment for bone regeneration is

successful when the scaffold is capable of stimulating and regulating cellular, vascular and immune responses to restore the functionality of the bone. In addition to the similarity of the material with bone structure, these scaffolds should also promote osteogenesis and diminish the negative effects of inflammatory reactions. Data is accumulating that the mineral matrix in which it is deposited and organized inside collagen is a key factor in determining these biological responses, which influence cell behavior, vascularization and immune modulation. The effective mineralized collagen scaffolds attract osteoblasts and angiogenesis in part by stimulating important signaling pathways such as the one which involves HIF-1/VEGF that encourages the development of new blood vessels essential to bone healing. Besides, they can regulate immune response by inducing phenotypes of macrophages which have anti-inflammatory and pro-healing effects and thus creating a microenvironment that supports regeneration. Therefore, it is important to optimize the mineralization pattern in collagen scaffolds to deliver a balance of structural integrity and biological function to achieve the best outcome for bone regeneration.

Cell adhesion, proliferation, and osteogenic differentiation

Apatite-based osteoconductive scaffolds *in situ* mineralized with collagen result in a biologically preferred interface that combines the natural cell-binding sites of collagen with the apatite. Nanoscale mineral increases the adsorption of adhesion proteins, such as fibronectin and vitronectin, that promotes integrin-mediated cell attachment. These scaffolds are consistently superior in promoting mesenchymal stem cell (MSC) adhesion, proliferation, and osteogenic differentiation, as indicated by enhanced expression of the markers ALP, RUNX2, OCN and COL1A1 compared with non-mineralized collagen or particle-filled composites [88]. More specifically, intrafibrillar and finely dispersed mineral phases are found to be more effective in inducing osteogenic signaling than coarse and surface-deposited minerals, presumably due to their greater similarity to the native bone extracellular matrix structure. This indicates that the biological performance of mineralized collagen scaffolds lies more in the nanoarchitecture of the mineral itself as opposed to the total amount. Thus, it is important to optimize mineral nanoarchitecture to achieve improved bioactivity of scaffolds and to promote bone regeneration.

Osteoimmunomodulation and inflammatory reaction

Close connections between bone regeneration and immune regulation imply that initial inflammatory reactions are extremely important in the healing process. When mineral deposition is properly regulated, *in situ* mineralized collagen scaffolds can modulate macrophage behavior by inducing polarization to a pro-regenerative M2-like phenotype. Biomimetic mineral structures down-regulate the expression of pro-inflammatory cytokines like TNF- α and IL-1 β as well as increasing the release of factors which promote angiogenesis and osteogenesis [85, 89]. Nonetheless, the overabundance or unchecked extrafibrillar mineralization can result in a foreign-body response and chronic inflammation that can impair bone healing. These results underline the significance of the choice of the right mineralization strategies to influence the immune microenvironment in a positive way, and the proper bone regeneration process should be based on the coordination of osteogenesis and immunomodulation [90]. Hence, the mineral architecture optimization of collagen scaffolds is necessary to balance the immune reactions and ensure functional bone regeneration.

Bone Formation and vascularization in vivo

The process of bone regeneration is strongly connected to immune regulation, and initial inflammatory reactions are critical in the healing responses. When polarization toward a more pro-regenerative M2-like phenotype is desired, *in situ* mineralized collagen scaffolds can regulate the behaviour of macrophages by tightly regulating mineral deposition. Biomimetic mineral networks decrease the production of pro-inflammatory cytokines like TNF- α and IL-1 β , and increase the release of angiogenic and osteogenic-supportive factors [85]. Nevertheless, it is possible that an excess or improperly controlled extrafibrillar mineralization will initiate foreign-body responses and chronic inflammation, which will interfere with bone repair. These results have reinforced the necessity of using the right mineralization techniques to influence the immune microenvironment in a positive manner, and that efficient bone regeneration needs coordinated osteogenesis and immunomodulation [90]. In that way, it is necessary to optimize mineral architecture in collagen scaffolds to balance the immune response and enhance functional bone healing.

Compared to other Mineralization Approaches

Collagen-based scaffolds may be mineralized *in situ*, *ex situ* by incorporation of nanoparticles, and by surface coating, all of which vary in their capacity to

recapitulate the hierarchical structure and function of bone. Mineral phase that is mineralized *in situ* within the collagen matrix allows the mineral nucleation, distribution, and orientation to be controllable, in particular to achieve intrafibrillar mineralization; this leads to enhanced organic-inorganic integration, greater toughness, and more biological behavior but must be optimized to ensure reproducibility and scalability [16]. *Ex situ* nanoparticle blending consists of incorporating pre-formed hydroxyapatite or calcium phosphate particles into collagen scaffolds; although technically simpler, and scalable, it can result in random extrafibrillar distribution of the mineral, with weak bonds and possible agglomeration of particles, reducing mechanical efficacy and biomimicry [78].

Surface coating techniques enhance scaffold bioactivity by changing the surface characteristics via apatite deposition or plasma spraying but do not enhance bulk scaffold mechanics and can be easily delaminated or inadequately covered, which restricts control over hierarchical mineral organization and remodelling [91]. In general, hierarchical integration of mineral and collagen made *in situ* is the only method that closely mimics the structure and functioning of native bone and is the most promising method despite the manufacturing difficulties relative to simpler approaches. The strategy adopted is determined by a tradeoff between biomimetic fidelity and more feasible factors such as scalability and potential clinical translation [92].

Barriers to manufacturing and Translation

Developing *in situ* mineralized collagen scaffolds have been associated with huge challenges related to production, sterilization, mechanical testing, and regulatory barriers. The sensitivity of the mineralization processes to factors such as ion concentration, pH, and temperature is a barrier to manufacturing reproducibility, leaving batch variability and scaling up complex scaffold shapes to good manufacturing practice (GMP) requirements. The gamma irradiation or autoclaving of collagen can destroy collagen structure and mineral phases, leading to impaired scaffold integrity and biological activity; nonetheless, sterilization-compatible methods are understudied. There is a lack of mechanical reliability and long-term performance information and most studies have concentrated on short-term results in small-animal models as opposed to load-bearing conditions or cyclic loading conditions of clinical use [92]. The regulatory classification depends on the scaffold structure and additives, making it more difficult to obtain approval, and high production costs and short shelf life make it even harder to commercialize in comparison to proven bone graft substitutes. These

obstacles will have to be overcome with intensive manufacturing guidelines, standardized testing systems with long-term big-animal trials and definite clinical benchmarking with existing bone repair systems to provide effective clinical uptake.

Open Questions and Ongoing Debates in the Field

Beyond the specific translational barriers already discussed, several unresolved specific questions run through the literature reviewed in this article and deserve to be named explicitly rather than left implicit. First, the extent to which the amorphous-precursor pathway documented directly *in vivo* in a small number of model systems [58, 59] generalizes across mammalian long-bone formation, and across the many *in situ* mineralization protocols in this review that assume it, it remains an open empirical question rather than a settled mechanism; most collagen scaffold studies infer ACP involvement indirectly from electron diffraction or spectroscopic signature rather than from the kind of time-resolved, spatially mapped evidence available in the zebrafish fin-ray model. Second, essentially none of the *in situ* mineralization methods surveyed here, whether SBF-based, ion-mediated, enzyme-mediated or PILP-based, report the water content or hydration state of the resulting scaffold, despite the evidence that water is a mechanically active, independent constituent of native bone rather than inert filler [8, 10]; without this measurement, reported gains in scaffold stiffness or toughness cannot be fully attributed to mineral architecture as opposed to a confounding change in matrix hydration during processing. Third, the SBF-realism critique discussed above versus the pragmatic case for SBF as a bioactivity screen [74] remains an active disagreement in the wider biomaterial's literature that the collagen-scaffold subfield has largely imported without resolving, since *in situ* mineralized collagen scaffolds are also most commonly first screened in SBF before any *in vivo* testing. Finally, and connecting back to the manufacturing barriers discussed above essentially all of the mechanistic and structure-function evidence in this review comes from short duration, small-specimen, non-load-bearing *in vitro* or small-animal studies, so the field's confidence in mechanisms such as intrafibrillar-mineralization driven toughness rests on a narrower evidence base, in terms of scaffold scale and physiological loading duration, than the strength of the underlying nanoscale sciences might suggest.

Future Views and Research Focuses

The advancement of *in situ* mineralized collagen scaffolds in the future will depend on the development of quantitative design principles that bridge mineralization processes, hierarchical

structure and functional performance, and a standardized measurement of intrafibrillar mineral content, mineral orientation and long-term mechanical stability to allow comparisons across studies [93]. It is important to highlight the need to focus on osteoimmunomodulation, vascularization and remodeling compatibility in clinically relevant disease models including osteoporosis and diabetes as a means of developing biological relevance. Scalability of manufacturing and compatibility with sterilization should be considered as early in development as possible and integration of newer fabrication methods such as 3D printing and controlled ion-delivery systems have potential to permit the design of patient-specific scaffolds with enhanced reproducibility. New approaches that involve synchronous self-assembly/mineralization or polymer-induced liquid precursors have proven to be effective in improving the scaffold homogeneity, mechanical strength, and osteogenic potential [78]. They still need to be tested in long-term large-animal models and direct comparisons against existing clinical standards to confirm long-term durability, remodeling behavior, and functional benefits during bone defect repair [94]. Altogether, the development of biomimetic mineralized collagen scaffolds into clinical practice will involve concerted actions in the field of materials design, manufacturing guidelines, biological testing, and regulatory affairs.

Conclusion

Mineralization of collagen-based scaffolds *in situ* provides a promising biomimetic technique for bone tissue engineering as it recapitulates the natural hierarchical organization of collagen and apatite in bone. This technique enables the accurate control of mineral nucleation, distribution and integration into the collagen matrix to exhibit increased mechanical strength and better biological responses than other conventional methods of mineralization. While these benefits are available, there are challenges that need to be addressed regarding the need for consistency in manufacturing processes, long-term mechanical stability and navigating complex regulatory pathways. These issues need to be dealt with successfully in order to go beyond laboratory work and create effective clinical solutions to repair large bone defects.

Declaration of Competing Interests

There are no competing interests to disclose.

Funding Declaration

No financial support was received by the authors for conducting, writing, or publishing this research.

Data Availability

No Research Data are Associated With this Article

References

- Lasanianos, N.G., N.K. Kanakaris, and P.V. Giannoudis, *Current management of long bone large segmental defects*. Orthopaedics and Trauma, 2010. **24**(2): p. 149-163.
- Nicholson, J.A., et al., *Fracture nonunion in long bones: A literature review of risk factors and surgical management*. Injury, 2021. **52 Suppl 2**: p. S3-s11.
- Gómez-Barrena, E. and C. Ehrnthaller, *Long bone uninfected non-union: grafting techniques*. EFORT Open Rev, 2024. **9**(5): p. 329-338.
- Maisenbacher, T., et al., *Direct and indirect costs of long bone fracture nonunions of the lower limb: the economic burden on the German healthcare system*. Bone & Joint Research, 2025. **14**: p. 341-350.
- Perez, J.R., et al., *Tissue Engineering and Cell-Based Therapies for Fractures and Bone Defects*. Front Bioeng Biotechnol, 2018. **6**: p. 105.
- Liu, Y., D. Luo, and T. Wang, *Hierarchical Structures of Bone and Bioinspired Bone Tissue Engineering*. Small, 2016. **12**(34): p. 4611-32.
- Nanda, R., et al., *Molecular differences in collagen organization and in organic-inorganic interfacial structure of bones with and without osteocytes*. Acta Biomaterialia, 2022. **144**: p. 195-209.
- Boskey, A.L., *Bone composition: relationship to bone fragility and antiosteoporotic drug effects*. Bonekey Rep, 2013. **2**: p. 447.
- Glimcher, M.J., *The nature of the mineral component of bone and the mechanism of calcification*. Instr Course Lect, 1987. **36**: p. 49-69.
- Granke, M., M.D. Does, and J.S. Nyman, *The Role of Water Compartments in the Material Properties of Cortical Bone*. Calcif Tissue Int, 2015. **97**(3): p. 292-307.
- Unal, M., S. Yang, and O. Akkus, *Molecular spectroscopic identification of the water compartments in bone*. Bone, 2014. **67**: p. 228-36.
- Ulian, G., D. Moro, and G. Valdrè, *Hydroxylapatite and Related Minerals in Bone and Dental Tissues: Structural, Spectroscopic and Mechanical Properties from a Computational Perspective*. Biomolecules, 2021. **11**(5).
- Landis, W.J., *The strength of a calcified tissue depends in part on the molecular structure and organization of its constituent mineral crystals in their organic matrix*. Bone, 1995. **16**(5): p. 533-44.
- Baldwin, P., et al., *Autograft, Allograft, and Bone Graft Substitutes: Clinical Evidence and Indications for Use in the Setting of Orthopaedic Trauma Surgery*. J Orthop Trauma, 2019. **33**(4): p. 203-213.
- Fernandez de Grado, G., et al., *Bone substitutes: a review of their characteristics, clinical use, and perspectives for large bone defects management*. J Tissue Eng, 2018. **9**: p. 2041731418776819.
- Shi, R., et al., *Collagen-mediated in situ mineralization-enhanced biomimetic bone tissue engineering scaffolds*. Colloids and Surfaces B: Biointerfaces, 2025. **250**: p. 114566.
- Schlesinger, P.H., et al., *Cellular and extracellular matrix of bone, with principles of synthesis and dependency of mineral deposition on cell membrane transport*. Am J Physiol Cell Physiol, 2020. **318**(1): p. C111-c124.
- Wingender, B., et al., *Biomimetic organization of collagen matrices to template bone-like microstructures*. Matrix Biol, 2016. **52-54**: p. 384-396.
- Xu, Y., et al., *Intermolecular channels direct crystal orientation in mineralized collagen*. Nature Communications, 2020. **11**(1): p. 5068.
- Li, Z., et al., *Bioinspired mineralized collagen scaffolds for bone tissue engineering*. Bioactive Materials, 2021. **6**(5): p. 1491-1511.
- Koushik, T.M., C.M. Miller, and E. Antunes, *Bone Tissue Engineering Scaffolds: Function of Multi-Material Hierarchically Structured Scaffolds*. Adv Healthc Mater, 2023. **12**(9): p. e2202766.
- Zhu, L., D. Luo, and Y. Liu, *Effect of the nano/microscale structure of biomaterial scaffolds on bone regeneration*. Int J Oral Sci, 2020. **12**(1): p. 6.
- Weiner, S. and D. Wagner, *THE MATERIAL BONE: Structure-Mechanical Function Relations*. Annual Review of Materials Science, 2003. **28**: p. 271-298.
- Rico-Llanos, G.A., et al., *Collagen Type I Biomaterials as Scaffolds for Bone Tissue Engineering*. Polymers (Basel), 2021. **13**(4).
- Nogueira, L.F.B., et al., *Collagen/k-Carrageenan-Based Scaffolds as Biomimetic*

- Constructs for In Vitro Bone Mineralization Studies*. *Biomacromolecules*, 2023. **24**(3): p. 1258-1266.
26. Selvaraj, V., et al., *Type I collagen: Synthesis, structure and key functions in bone mineralization*. *Differentiation*, 2024. **136**: p. 100757.
27. Hodge, A.J. and J.A. Petruska. *Recent studies with the electron microscope on ordered aggregates of the tropocollagen macromolecule*. 1963.
28. Traub, W., T. Arad, and S. Weiner, *Three-dimensional ordered distribution of crystals in turkey tendon collagen fibers*. *Proc Natl Acad Sci U S A*, 1989. **86**(24): p. 9822-6.
29. Yu, L. and M. Wei, *Biom mineralization of Collagen-Based Materials for Hard Tissue Repair*. *Int J Mol Sci*, 2021. **22**(2).
30. Hu, C., L. Zhang, and M. Wei, *Development of Biomimetic Scaffolds with Both Intrafibrillar and Extrafibrillar Mineralization*. *ACS Biomater Sci Eng*, 2015. **1**(8): p. 669-676.
31. Wang, Y., et al., *Synergistic intrafibrillar/extrafibrillar mineralization of collagen scaffolds based on a biomimetic strategy to promote the regeneration of bone defects*. *Int J Nanomedicine*, 2016. **11**: p. 2053-67.
32. Carvalho, M.S., et al., *Bone Matrix Non-Collagenous Proteins in Tissue Engineering: Creating New Bone by Mimicking the Extracellular Matrix*. *Polymers (Basel)*, 2021. **13**(7).
33. Liu, X., et al., *A novel osteopontin biomimetic polypeptide induces collagen fiber mineralization in vitro*. *International Journal of Biological Macromolecules*, 2025. **310**: p. 143245.
34. Gleeson, S.E., et al., *Insight on the Role of Poly(acrylic acid) for Directing Calcium Phosphate Mineralization of Synthetic Polymer Bone Scaffolds*. *ACS applied bio materials*, 2022.
35. Su, W., et al., *Alginate-Assisted Mineralization of Collagen by Collagen Reconstitution and Calcium Phosphate Formation*. *ACS Biomater Sci Eng*, 2020. **6**(6): p. 3275-3286.
36. Hua, R., et al., *Pivotal roles of biglycan and decorin in regulating bone mass, water retention, and bone toughness*. *Bone Res*, 2025. **13**(1): p. 2.
37. Terzi, A., et al., *Sub- and Supramolecular X-Ray Characterization of Engineered Tissues from Equine Tendon, Bovine Dermis, and Fish Skin Type-I Collagen*. *Macromol Biosci*, 2020. **20**(5): p. e2000017.
38. Sallent, I., et al., *Assessing the Potential of Caprine Collagen Type I in the Development of Medical Devices*. *Biomacromolecules*, 2025. **26**(10): p. 6418-6429.
39. Liu, S., et al., *Marine collagen scaffolds in tissue engineering*. *Current Opinion in Biotechnology*, 2022. **74**: p. 92-103.
40. Maher, M., et al., *Suitability of Marine- and Porcine-Derived Collagen Type I Hydrogels for Bioprinting and Tissue Engineering Scaffolds*. *Mar Drugs*, 2022. **20**(6).
41. Coppola, A., et al., *Marine Collagen from European Sea Bass (*Dicentrarchus labrax*) Waste for the Development of Chitosan/Collagen Scaffolds in Skin Tissue Engineering*. *Mar Drugs*, 2025. **23**(10).
42. Yang, Y., et al., *Development of Recombinant Human Collagen-Based Porous Scaffolds for Skin Tissue Engineering: Enhanced Mechanical Strength and Biocompatibility*. *Polymers (Basel)*, 2025. **17**(3).
43. Diogo, G.S., et al., *Cell-Laden Biomimetically Mineralized Shark-Skin-Collagen-Based 3D Printed Hydrogels for the Engineering of Hard Tissues*. *ACS Biomater Sci Eng*, 2020. **6**(6): p. 3664-3672.
44. Griffanti, G., M.D. McKee, and S.N. Nazhat, *Mineralization of Bone Extracellular Matrix-like Scaffolds Fabricated as Silk Sericin-Functionalized Dense Collagen-Fibrin Hybrid Hydrogels*. *Pharmaceutics*, 2023. **15**(4).
45. Rocha, M.S., et al., *3D-Printed Cell-Instructive Scaffolds Based on Chondrosia reniformis Collagen and Sr-Doped Calcium Phosphates for Bone Tissue Engineering*. *ACS Biomater Sci Eng*, 2025. **11**(6): p. 3547-3559.
46. Krishnakumar, G.S., et al., *Evaluation of different crosslinking agents on hybrid biomimetic collagen-hydroxyapatite composites for regenerative medicine*. *Int J Biol Macromol*, 2018. **106**: p. 739-748.
47. Elias, J., B.-A. Matheson, and L. Gower, *Influence of Crosslinking Methods on Biomimetically Mineralized Collagen Matrices for Bone-like Biomaterials*. *Polymers*, 2023. **15**(9): p. 1981.
48. Pawelec, K.M., et al., *Osteogenesis and mineralization of mesenchymal stem cells in collagen type I-based recombinant peptide scaffolds*. *J Biomed Mater Res A*, 2017.

- 105(7):** p. 1856-1866.
49. Bonar, L.C., et al., *X-ray diffraction studies of the crystallinity of bone mineral in newly synthesized and density fractionated bone*. *Calcif Tissue Int*, 1983. **35(2)**: p. 202-9.
50. Wang, L. and G.H. Nancollas, *Pathways to biomineralization and biode mineralization of calcium phosphates: the thermodynamic and kinetic controls*. *Dalton Trans*, 2009(15): p. 2665-72.
51. LeGeros, R.Z., *Calcium phosphates in oral biology and medicine*. *Monogr Oral Sci*, 1991. **15**: p. 1-201.
52. Kim, D., et al., *The role of confined collagen geometry in decreasing nucleation energy barriers to intrafibrillar mineralization*. *Nat Commun*, 2018. **9(1)**: p. 962.
53. Kim, D., et al., *In Situ Evaluation of Calcium Phosphate Nucleation Kinetics and Pathways during Intra- and Extrafibrillar Mineralization of Collagen Matrices*. *Cryst Growth Des*, 2016. **16(9)**: p. 5359-5366.
54. Olszta, M.J., E.P. Douglas, and L.B. Gower, *Scanning electron microscopic analysis of the mineralization of type I collagen via a polymer-induced liquid-precursor (PILP) process*. *Calcif Tissue Int*, 2003. **72(5)**: p. 583-91.
55. Ma, Y.-x., et al., *Involvement of prenucleation clusters in calcium phosphate mineralization of collagen*. *Acta Biomaterialia*, 2021. **120**: p. 213-223.
56. Zhu, Q., et al., *Hydroxyapatite formation in biomimetic synthesis with the interface of a pDA@SIS membrane*. *RSC Adv*, 2022. **12(21)**: p. 13209-13219.
57. Qin, D., et al., *Liquid-Liquid Phase Separation in Nucleation Process of Biomineralization*. *Front Chem*, 2022. **10**: p. 834503.
58. Mahamid, J., et al., *Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: Indications for an amorphous precursor phase*. *Proc Natl Acad Sci U S A*, 2008. **105(35)**: p. 12748-53.
59. Mahamid, J., et al., *Mapping amorphous calcium phosphate transformation into crystalline mineral from the cell to the bone in zebrafish fin rays*. *Proc Natl Acad Sci U S A*, 2010. **107(14)**: p. 6316-21.
60. Shen, D.N., et al., *Citrate Improves Biomimetic Mineralization Induced by Polyelectrolyte-Cation Complexes Using PAsp-Ca&Mg Complexes*. *Adv Healthc Mater*, 2024. **13(15)**: p. e2303870.
61. Mi, X., et al., *Three-Dimensional Electrodeposition of Calcium Phosphates on Porous Nanofibrous Scaffolds and Their Controlled Release of Calcium for Bone Regeneration*. *ACS Appl Mater Interfaces*, 2020. **12(29)**: p. 32503-32513.
62. Zhang, Y., et al., *Effect of the stability of the poly(gamma-glutamic acid)-ACP dispersion system on biomimetic mineralization of type I collagen*. *Biomater Sci*, 2025. **13(3)**: p. 795-809.
63. Nudelman, F., et al., *The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors*. *Nature Materials*, 2010. **9(12)**: p. 1004-1009.
64. Jiang, W., et al., *Multiscale structural evolution of citrate-triggered intrafibrillar and interfibrillar mineralization in dense collagen gels*. *J Struct Biol*, 2020. **212(1)**: p. 107592.
65. Chen, X., et al., *Fabrication and Biomedical Application of Alginate Composite Hydrogels in Bone Tissue Engineering: A Review*. *Int J Mol Sci*, 2024. **25(14)**.
66. Gouissem, A., F. Alkhatib, and M. Adouni, *Investigating the influence of mineral content changes on mechanical properties through ligament insertion*. *Front Aging*, 2025. **6**: p. 1556577.
67. El-Fiqi, A., J.-H. Kim, and H.-W. Kim, *Novel bone-mimetic nanohydroxyapatite/collagen porous scaffolds biomimetically mineralized from surface silanized mesoporous nanobioglass/collagen hybrid scaffold: Physicochemical, mechanical and in vivo evaluations*. *Materials Science and Engineering: C*, 2020. **110**: p. 110660.
68. Gieroba, B., et al., *Collagen maturity and mineralization in mesenchymal stem cells cultured on the hydroxyapatite-based bone scaffold analyzed by ATR-FTIR spectroscopic imaging*. *Mater Sci Eng C Mater Biol Appl*, 2021. **119**: p. 111634.
69. George, M. and T.E. Abraham, *Polyionic hydrocolloids for the intestinal delivery of protein drugs: Alginate and chitosan — a review*. *Journal of Controlled Release*, 2006. **114(1)**: p. 1-14.
70. Liu, Y., et al., *Intrafibrillar collagen mineralization produced by biomimetic hierarchical nanoapatite assembly*. *Adv Mater*, 2011. **23(8)**: p. 975-80.
71. Thrivikraman, G., et al., *Rapid fabrication of*

- vascularized and innervated cell-laden bone models with biomimetic intrafibrillar collagen mineralization. *Nature Communications*, 2019. **10**(1): p. 3520.
72. Bim-Júnior, O., et al., *Surface-Directed Mineralization of Fibrous Collagen Scaffolds in Simulated Body Fluid for Tissue Engineering Applications*. *ACS Appl Bio Mater*, 2021. **4**(3): p. 2514-2522.
73. Xia, Z., et al., *Fabrication and characterization of biomimetic collagen-apatite scaffolds with tunable structures for bone tissue engineering*. *Acta Biomater*, 2013. **9**(7): p. 7308-19.
74. Kokubo, T. and H. Takadama, *How useful is SBF in predicting in vivo bone bioactivity?* *Biomaterials*, 2006. **27**(15): p. 2907-2915.
75. Bohner, M. and J. Lemaître, *Can bioactivity be tested in vitro with SBF solution?* *Biomaterials*, 2009. **30**(12): p. 2175-2179.
76. Chen, J., et al., *Enzyme-Activated Biomimetic Vesicles Confining Mineralization for Bone Maturation*. *ACS Appl Mater Interfaces*, 2024. **16**(26): p. 33005-33020.
77. Murshed, M., *Mechanism of Bone Mineralization*. *Cold Spring Harb Perspect Med*, 2018. **8**(12).
78. Li, Z., et al., *In-situmineralized homogeneous collagen-based scaffolds for potential guided bone regeneration*. *Biofabrication*, 2022. **14**(4).
79. Yuan, Z., et al., *Controlled magnesium ion delivery system for in situ bone tissue engineering*. *Journal of Controlled Release*, 2022. **350**: p. 360-376.
80. Cui, X., et al., *Piezocatalytically-induced controllable mineralization scaffold with bone-like microenvironment to achieve endogenous bone regeneration*. *Science Bulletin*, 2024. **69**(12): p. 1895-1908.
81. Niu, Y., et al., *Compressive stress improves mechanical properties of mineralized collagen by dynamically regulating its mineralization - a closed-loop regulation mechanism*. *Materials & Design*, 2024. **239**: p. 112830.
82. Liu, Y., et al., *Effect of nanostructure of mineralized collagen scaffolds on their physical properties and osteogenic potential*. *J Biomed Nanotechnol*, 2014. **10**(6): p. 1049-60.
83. Sadowska, J.M., et al., *Effect of nano-structural properties of biomimetic hydroxyapatite on osteoimmunomodulation*. *Biomaterials*, 2018. **181**: p. 318-332.
84. Yi, L., et al., *Tuning the Intrafibrillar Collagen Mineralization Rate and Mechanical Properties Through Polyelectrolyte-Controlled Formation and Crystallization of Amorphous Precursor*. *Small*, 2025. **21**(16): p. e2411443.
85. Jin, S.S., et al., *A Biomimetic Hierarchical Nanointerface Orchestrates Macrophage Polarization and Mesenchymal Stem Cell Recruitment To Promote Endogenous Bone Regeneration*. *ACS Nano*, 2019. **13**(6): p. 6581-6595.
86. Cui, Y., et al., *Novel insights into nanomaterials for immunomodulatory bone regeneration*. *Nanoscale Adv*, 2022. **4**(2): p. 334-352.
87. Nicolas, T., et al., *Multiparametric influence of 3D-printed organo-mineral scaffolds on bone regeneration*. *Sci Rep*, 2024. **14**(1): p. 20848.
88. Grue, B.H. and S.P. Veres, *Effect of increasing mineralization on pre-osteoblast response to native collagen fibril scaffolds for bone tissue repair and regeneration*. *J Appl Biomater Funct Mater*, 2022. **20**: p. 22808000221104000.
89. Zhou, Y., et al., *Intrafibrillar Mineralization and Immunomodulatory for Synergetic Enhancement of Bone Regeneration via Calcium Phosphate Nanocluster Scaffold*. *Adv Healthc Mater*, 2023. **12**(12): p. e2201548.
90. Abedi, N., et al., *Immunomodulation in Bone Tissue Engineering: Recent Advancements in Scaffold Design and Biological Modifications for Enhanced Regeneration*. *ACS Biomater Sci Eng*, 2025. **11**(3): p. 1269-1290.
91. Santhakumar, S., et al., *Bone Tissue Regeneration by Collagen Scaffolds with Different Calcium Phosphate Coatings: Amorphous Calcium Phosphate and Low-Crystalline Apatite*. *Materials*, 2021. **14**(19): p. 5860.
92. Zhu, W., et al., *Mineralized Collagen/Polylactic Acid Composite Scaffolds for Load-Bearing Bone Regeneration in a Developmental Model*. *Polymers (Basel)*, 2023. **15**(20).
93. Wu, B., et al., *Biomimetic Mineralized Collagen Scaffolds for Bone Tissue Engineering: Strategies on Elaborate Fabrication for Bioactivity Improvement*. *Small*, 2025. **21**(3): p. e2406441.
94. Yu, L., et al., *Intrafibrillar Mineralized Collagen-Hydroxyapatite-Based Scaffolds for Bone Regeneration*. *ACS Appl Mater*

Interfaces, 2020. **12**(16): p. 18235-18249.