From bone regeneration to three-dimensional in vitro models: tissue engineering of organized bone extracellular matrix

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Abstract
Traditionally, bone tissue engineering has been used for creating implants for bone regeneration, but recently, it is increasingly applied to create three-dimensional (3D) in vitro bone models to study bone physiology and pathology. For 3D in vitro bone models, the engineered extracellular matrix organization should resemble the in vivo physiological bone structure because this is often the hallmark in skeletal pathologies — an issue that has not been solved yet. In this review article, we define the extracellular matrix requirements for an optimal 3D in vitro model based on the most recent advances on bone structure. To meet these requirements, osteoclasts, osteocytes, and mature osteoblasts should work together under physiological conditions, and the formed extracellular matrix should be analyzed and optimized at multiple length scales.

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Keywords
Bone tissue engineering, In vitro model, Extracellular matrix, Mineralization, Collagen organization.

Abbreviations
ECM, extracellular matrix; FBS, fetal bovine serum; MSCs, mesenchymal stromal cells; NCPs, noncollagenous proteins; DMP1, dentin matrix protein 1.

Introduction
Bones have remarkable mechanical properties thanks to their extraordinary extracellular matrix (ECM) composition and organization [1]. To attain these mechanical properties, organic and inorganic matrix components are highly organized at multiple hierarchical levels [2] and continuously remodeled by osteoclasts (bone-resorbing cells), osteoblasts (bone-forming cells), and osteocytes (regulating cells) [3,4]. During the last few decades, researchers have attempted to mimic bone using a tissue engineering approach.

Traditionally, bone tissue engineering has been focusing on developing grafts for patients with large osseous defects, making use of scaffolds, progenitor cells, mechanical stimuli, and soluble factors. For bone regeneration, this approach was used to achieve constructs with osteogenic, osteoconductive, and osteoinductive properties [5]. As such, tissue-engineered bone-like constructs are able to induce regeneration, even if they fail to resemble the complex ECM structure of real human bone, thanks to bone’s innate capacity to regenerate and remodel. Over the last few years, bone tissue engineering is increasingly applied to develop three-dimensional (3D) in vitro human bone models for drug testing, addressing the principle of reduction, refinement, and replacement [6,7]. For 3D in vitro bone models, the engineered ECM structure should resemble the complex ECM structure of physiological human bone. This is particularly important for models aiming at mimicking bone pathologies where remodeling is affected, as in these situations the bone’s ECM composition and organization, and thus its mechanical competence is often changed [8]. For example, in osteogenesis imperfecta, the collagen network is more loosely woven and minerals appear smaller and disorganized, resulting in brittle bones [9]. Another example is osteoporosis, where collagen fibrils were found to be less aligned and only partly mineralized in a mouse model, leading to fragile bones [10]. To be able to mimic the ECM structure of these pathologies and to distinguish it from the healthy
situation, the *in vitro* recreation of the healthy bone ECM structure should be accomplished first.

With this review article, we attempt to define what to aim for in bone tissue engineering when creating 3D *in vitro* models representing healthy bone physiology. In this regard, we (i) characterize the physiological human *in vivo* bone structure, (ii) give recommendations on criteria for an *in vitro* model for physiological human bone, (iii) discuss what has been achieved thus far in meeting these criteria making use of bone tissue engineering, (iv) suggest how we might optimize the ECM structure for *in vitro* bone models to meet the proposed criteria in future, and (v) propose how these criteria should be assessed.

**The organic matrix: a template for mineralization**

Bone ECM consists of ~30–40% of organic matrix that provides bone with toughness and elastic properties [3]. The organic matrix is mainly composed of collagen type I molecules, produced by osteoblasts [3]. These molecules form triple helices that can assemble into collagen microfibrils in a twisted and staggered arrangement, with a gap region between two consecutive molecules [11]. Multiple collagen microfibrils can bundle into collagen fibrils, and in turn, multiple collagen fibrils can form a collagen fiber (Figure 1a). The orientation of these collagen fibers contributes to the mechanical competence of bone [12]. More specifically, collagen networks that are aligned parallel to the load are better resistant to tension, whereas collagen networks aligned perpendicular to the load are better resistant to compression [13,14]. Collagen network density and fibril organization are important determinants for successful subsequent mineralization [15,16] (Section 3). Based on the organization of collagen, we can describe two extremes of bone structure organization (although more gradations can be made [2]): (i) the more immature woven bone with a disordered collagen fiber structure and loosely packed poorly oriented collagen fibrils and (ii) the more mature lamellar bone with a dense network of parallel aligned collagen fibers within a lamella but with alternating orientation between lamellae [17]. *In vivo*, woven bone is thought to be the primary formed bone by osteoblasts that rapidly secrete collagen which assembles into fibril bundles with little or no preferred orientation [2,18]. Lamellar bone is formed upon bone remodeling; it is believed that osteoblasts array themselves in a polarized fashion along a surface and secrete collagen fibrils onto the surface in a parallel orientation [18]. A small portion of the organic matrix is composed of noncollagenous proteins (NCPs) [3], which are believed to play an important role in collagen fibril assembly and subsequent mineralization of these collagen fibrils [19,20]. Together, the collagen network, individual collagen fibril organization, and NCPs form a template for mineralization and are thus a major determinant for the eventual ECM structure. Therefore, to resemble physiological bone in 3D *in vitro* bone models, the organic matrix should comprise a highly dense and aligned collagen network at the micrometer scale and highly organized collagen fibrils at the nanometer scale (Figure 1). NCPs are instrumental in the collagen organization and facilitate its mineralization.

For *in vitro* bone tissue engineering, collagen network density and orientation are not common outcome parameters. When visualized with conventional light microscopy, the collagen networks seen in *in vitro* engineered constructs are often at a low level of organization, which can in the best cases be characterized as similar to the immature woven bone. In addition, because these collagen networks are often only assessed at the micrometer scale, it is unclear whether the individual collagen fibrils exhibit uniformly a high level of organization. Anatomic-level evaluation of collagen produced by osteoblasts *in vitro* and *in vivo* showed misfolded collagen proteins in the two-dimensional *in vitro* situation [21]. It was suggested that collagen protein folding could be improved by optimization of the culture conditions [21], emphasizing the importance of multiscale analysis when optimizing protocols that aim for the resemblance of *in vivo* bone structures. Moreover, although important for collagen fibril assembly and subsequent mineralization, the function of NCPs is often neglected in bone tissue engineering. When studies report expression and/or synthesis of NCPs such as osteocalcin and alkaline phosphatase, they often use it to demonstrate the differentiation of the progenitor cells toward the osteogenic lineage instead of reporting on their distribution in the matrix and functions.

In recent years, researchers have attempted to create lamellar-like collagen networks with and without the use of cells. These studies are based on the concept that collagen alignment can be induced by collagen-producing cells, self-assembling collagen molecules, or selective collagen degradation [22]. More specifically, osteoblasts may influence collagen orientation by (i) exerting traction forces on their ECM [23] or (ii) by producing collagen parallel to their orientation, which can be guided by an oriented substrate [24,25] or mechanical stimuli such as stretch and fluid flow [26,27] where cells align to the surface or the direction of the applied mechanical stimulus. Thus far, these techniques have led to orientation of collagen in only one direction instead of an alternating orientation between collagen layers as present in lamellar bone *in vivo*. To create acellular dense collagen networks with alternating orientations between different collagen layers, collagen densification techniques were used [15,28–30]. These techniques are based on liquid crystal phasing of
collagen, a physiological state between liquid and solid in which highly concentrated collagen molecules can arrange and assemble [31]. In turn, such a dense and aligned collagen film can increase osteoblast proliferation and differentiation and guide the cells to align in the direction of the substrate collagen [32]. Under more physiological collagen concentrations, it has been demonstrated that collagen fibrils in solution can already align with the direction of flow in the absence of cells [33]. These aligned collagen fibrils were subsequently stabilized under physiological relevant strain (i.e. mimicking the traction forces of fibroblasts on ECM) to form a highly oriented collagen fiber [33]. ECM strain is not only important for the formation of oriented collagen fibers but can also protect already-existing fibrils, that are oriented in the direction of the strain, against degradation [34].

Although the aforementioned studies provide insight into the mechanisms of collagen alignment in bone, translation to 3D in vitro tissue engineering remains challenging. Studies that made use of substrates were all performed two-dimensionally and investigated cellular behavior over a short period of time, thus lacking spatial and temporal complexity. In addition, the use of collagen substrates to induce cell and collagen alignment in 3D is challenging, and degradation of these templates can result in collagen reorganization over time.

The organic matrix should comprise of organized collagen fibrils on the nanometer scale and a highly dense and aligned collagen fiber network on the micrometer scale. (a) Simplified representation of the collagen structure at multiple hierarchical levels. (b) Electron microscopy image of rat cortical bone collagen fibrils in vivo showing a high level of organization (reprinted from Bone, 26:4, Kafantari et al. Structural alterations in rat skin and bone collagen fibrils induced by ovariectomy, 349–353, Copyright (2000), with permission from Elsevier). (c) Second harmonic generation image of human femoral cortical bone showing a dense and aligned collagen fiber network (reprinted from Sci. Rep., 7:3419, Genthial et al., Label-free imaging of bone multiscale porosity and interfaces using third-harmonic generation microscopy, Copyright (2017), open access).
minerals (Figure 2a) [38]. Together these minerals form a continuous pattern of intrafibrillar and extrafibrillar dimensions of the single collagen fibril, forming a recently investigated, these minerals grow outside the c-axes parallel to the long axis of collagen fibrils. As hydroxyapatite crystals nucleate and orientate with their cursors enter the collagen gap regions where spaces.

To improve in vitro mineralization of collagen, it is important to know what factors can influence mineralization and how we can apply them in bone tissue engineering. Biochemically, citrate infiltration of collagen fibrils might promote intrafibrillar mineralization [41]. In addition, it is believed that citrate plays an important role in connecting individual mineral particles [42]. Citrate might be provided by osteoblasts that attain the ability to produce it during differentiation from mesenchymal stromal cells (MSCs) [43,44]. In addition, NCPs such as osteocalcin and osteopontin are believed to play an essential role in intrafibrillar crystal growth and crystal morphology [45]. Of importance, in vitro mineralization might be influenced by fetal bovine serum (FBS), a nutritional serum supplement often used for bone tissue engineering. The chemical composition of FBS is often not provided and varies from batch to batch and among different brands. Some FBS types even induce mineralization in the absence of cells [46]. In this way, mineralization may occur before differentiating cells can take charge over their formation, which might lead to uncontrolled mineral precipitation. To enable cell-controlled mineralization, medium composition needs to be optimized. Although the actual FBS components that influence mineralization are not known, alkaline phosphatase and fetuin likely play a role [19]. Both proteins not only are produced by osteoblasts but also may be present in FBS. More specifically, alkaline phosphatase can induce mineralization in the presence of calcium and the osteogenic culture supplement glycerophosphate [47], possibly leading to uncontrolled mineral precipitation in the extracellular spaces.

The collagen network is likely important for mineralization. It not only functions as a passive template; collagen fibril organization can improve mineralization by providing confined spaces at gap regions. These spaces might guide the minerals into the collagen fibrils [16]. Biomechanically, fluid shear stress can influence NCP expression/synthesis by osteoblasts [48], which will likely influence mineralization as well. As such, the magnitude of fluid flow–induced shear stresses has been shown to be predictive of mineral formation and might direct MSCs to proliferate or become osteoblasts, associated with increased mineralization [49,50]. These studies typically focus on mineral quantity instead of quality; thus, whether these fluid shear forces affect only the amount of mineralization or also mineral location with respect to the collagen fibrils, as characterized by Reznikov et al. [38], should be investigated. To investigate whether engineered mineralized bone ECM exhibits a highly mineralized collagen network with interconnected intrafibrillar and extrafibrillar minerals, tissue-engineered constructs and its individual mineralized collagen fibrils should be visualized at the nanometer scale.

The inorganic matrix: controlling intrafibrillar and extrafibrillar mineralization

The inorganic matrix of bone comprises ~60–70% of the total bone ECM and contains mainly carbonated hydroxyapatite [3]. In vivo, collagen mineralization appears inside and outside collagen fibrils, known as intrafibrillar and extrafibrillar mineralization, respectively [36,37]. Mineralization starts when mineral precursors enter the collagen gap regions where hydroxyapatite crystals nucleate and orientate with their c-axes parallel to the long axis of collagen fibrils. As recently investigated, these minerals grow outside the dimensions of the single collagen fibril, forming a continuous pattern of intrafibrillar and extrafibrillar minerals (Figure 2a) [38]. Together these minerals form an interconnected network in vivo [39], providing bone with mechanical rigidity and compressive strength [40]. Therefore, to resemble physiological human bone in 3D in vitro bone models, the inorganic matrix should comprise of a highly mineralized collagen network with interconnected intrafibrillar and extrafibrillar minerals (Figure 2).

Mineral organization is thus highly complex but unfortunately often not considered in bone tissue engineering. Instead, in bone tissue engineering, mineral deposition is often evaluated using calcium assays, calcium staining, or micro–computed tomography. These techniques say something about mineral content but not their specific size and location within the tissue, which are important for bone’s mechanical functionality [1]. It remains therefore unreported whether a bone tissue-engineered construct really comprises a mineralized collagen network, and if so, whether these minerals are located within the collagen fibrils, aligned on the outside, or only precipitated within the extracellular spaces.

To improve in vitro mineralization of collagen, it is important to know what factors can influence mineralization and how we can apply them in bone tissue engineering. Biochemically, citrate infiltration of collagen fibrils might promote intrafibrillar mineralization [41]. In addition, it is believed that citrate plays an important role in connecting individual mineral particles [42]. Citrate might be provided by osteoblasts that attain the ability to produce it during differentiation from mesenchymal stromal cells (MSCs) [43,44]. In addition, NCPs such as osteocalcin and osteopontin are believed to play an essential role in intrafibrillar crystal growth and crystal morphology [45]. Of importance, in vitro mineralization might be influenced by fetal bovine serum (FBS), a nutritional serum supplement often used for bone tissue engineering. The chemical composition of FBS is often not provided and varies from batch to batch and among different brands. Some FBS types even induce mineralization in the absence of cells [46]. In this way, mineralization may occur before differentiating cells can take charge over their formation, which might lead to uncontrolled mineral precipitation. To enable cell-controlled mineralization, medium composition needs to be optimized. Although the actual FBS components that influence mineralization are not known, alkaline phosphatase and fetuin likely play a role [19]. Both proteins not only are produced by osteoblasts but also may be present in FBS. More specifically, alkaline phosphatase can induce mineralization in the presence of calcium and the osteogenic culture supplement glycerophosphate [47], possibly leading to uncontrolled mineral precipitation in the extracellular spaces.
The inorganic matrix should comprise of a highly mineralized collagen network on the micrometer scale, and interconnected intrafibrillar and extrafibrillar minerals on the nanometer scale. (a) Simplified representation of the mineral structure, location of nucleation, and mineral growth. (b) Transmission electron microscopy image of mineralized collagen fibril showing a high level of alignment of intrafibrillar and extrafibrillar minerals (reprinted from Biomacromolecules, 12:8, A.S. Deshpande et al. Primary Structure and Phosphorylation of Dentin Matrix Protein 1 (DMP1) and Dentin Phosphophoryn (DPP) Uniquely Determine Their Role in Biomineralization, 2933–2945, Copyright (2011), with permission from American Chemical Society). (c) Scanning electron microscopy image of rat long bone showing mineralized aligned lamellae (reprinted from Acta Biomater., 10, Reznikov et al. Bone hierarchical structure in three dimensions, 3815–3826, Copyright (2014) [2], with permission from Elsevier).

**The cells: a role for osteoclasts, mature osteoblasts, and osteocytes?**

_In vitro_, organized bone matrices as characterized by Reznikov et al. [2] are only observed after remodeling which includes (i) activation or recruitment of progenitor cells, (ii) resorption of mineralized matrix by osteoclasts, (iii) surface preparation for bone formation, and (iv) ECM deposition by osteoblasts, regulated by osteocytes [49]. In contrast, the _in vitro_ approach usually starts with the differentiation of progenitor cells (mostly...
MSCs) toward matrix-producing osteoblasts, ignoring the contribution of osteocytes and osteoclasts. These progenitor cells start producing collagen and alkaline phosphatase during the initial stages of osteogenic differentiation [51]. This may lead to uncontrolled fibrillogenesis if no sufficient NCPs are produced at this stage, and, in the presence of serum supplemented medium, uncontrolled mineralization. Osteoblasts produce NCPs important for collagen fibril assembly and mineralization and develop the ability to produce citrate as they mature [43,51,52]. In addition, it has been demonstrated that undifferentiated MSC-like cells exert less traction forces on their substrate than mature osteoblast-like cells [23], indicating that mature osteoblasts have a superior ability to organize their ECM. Therefore, osteoblast maturation will likely improve ECM formation and organization as well.

When osteoblasts get embedded in their matrix, they can become osteocytes. Osteocytes are thought to guide bone formation and resorption by regulating the activities of osteoclasts and osteoblasts and are therefore important for overall bone structure [4]. Besides this regulation function, these cells produce proteins that are important for mineralization of collagen, for example, the NCP dentin matrix protein 1 [4,53]. Accordingly, osteocytes not only regulate overall bone structure but also influence the ECM organization at the nanometer scale, and they should therefore be included in 3D in vitro models for bone.

Recently, several osteoclast-derived factors that may influence osteoblast differentiation and subsequent matrix formation were identified [54,55]. In addition, because osteoclasts appear to deposit osteopontin in their resorption pits in vitro [56], they might have an active role in guiding mineralization. Moreover, synthesis of osteomodulin, important for fibrillogenesis, seems to be coupled to osteoclasts in vivo as its expression was reduced in osteoclast-deficient mice [52]. Thus, integration of osteoclasts not only may be important for resorption of the initially formed poorly organized ECM but also might improve ECM production by osteoblasts. Although in vitro bone remodeling by (mature) osteoblasts, osteocytes, and osteoclasts is still in its infancy (as reviewed by Owen and Reilly [7]), it might be the only way to achieve the formation of physiological bone ECM.

Cells thus play an important role in regulating the ECM organization by the factors they secrete. In turn, the ECM organization can influence the cellular behavior (Figure 3). For example, osteoclasts need a mineralized surface to form a proper actin ring to create a resorption pit. In addition, it is believed that osteoblasts in vivo only produce lamellar-like bone when they are in full contact with the solid substrate surface [18,57]. Accordingly, tissue engineering of 3D highly organized ECM is complex as the most effective starting point, that is, use an organized scaffold (as described by Liu et al. [58]) or let the cells organize their niche, is yet to be investigated.

Researchers have also attempted to improve osteogenesis by chondrogenic priming of progenitor cells [59]. This strategy is based on endochondral ossification, the mechanism by which long bones develop in vivo where a cartilage template is formed, mineralized, and subsequently replaced by woven bone. Endochondral ossification—based bone tissue engineering could benefit bone regeneration as it may improve angiogenesis and remodeling in vivo [59]. In vitro, chondrogenic priming might increase mineral content after subsequent osteogenic differentiation [60]. Whether this method could improve bone remodeling in vivo and thus potentially improve physiological bone formation is yet to be investigated.

Assessment of in vitro engineered bone-like structures

In bone tissue engineering, bone ECM components such as collagen, alkaline phosphatase, and minerals are often quantified and visualized at the micrometer scale to demonstrate osteogenesis. This might be enough for regeneration purposes, where, after implantation, the patient’s own cells can contribute to remodeling. However, with the goal to develop in vitro bone models that resemble the complex ECM of in vivo bone, additional structural assessments on both micrometer and nanometer scales are essential as explained in Section 2 and 3. In this regard, a multidisciplinary approach, in which bone biologists, material scientists, (bio)chemists, and mechanical engineers work together, should be taken to improve the formation and assessment of 3D in vitro engineered bone models. Based on the discussed topics in previous sections, we suggest assessing the following outcome parameters and propose techniques to assess them (Table 1). At the micrometer scale,
localization of minerals will require advanced techniques as well as at the nanometer scale, visualization of collagen fibrils, and mineral properties and location. Evaluation of collagen network orientation remains challenging because techniques based on polarization require a dense collagen network which is not always achieved in bone tissue engineering and which should be improved. As an alternative, fluorescent light microscopy images can be used in combination with a degree of orientation algorithm [61]. To assess whether the 3D in vitro bone model resembles physiological bone, these parameters should also be assessed and compared to ‘real’ physiological bone.

**Conclusion**

Taken together, with the purpose to create 3D in vitro bone models for studying bone-related diseases and drug testing, tissue-engineered ECM organization should resemble the in vivo physiological bone structure as this is often changed in pathological situations. We define the resemblance to the in vivo situation as a mineralized oriented dense collagen network with highly organized collagen fibrils and NCPs, allowing for intrafibrillar and organized extrafibrillar mineralization with interconnected mineral crystals. To compare the eventual ECM organization with the in vivo situation, researchers should consider assessing their in vitro bone models not only at the micrometer scale but also at the nanometer scale. To recreate the complex in vivo situation, the initially formed disorganized ECM might need to be resorbed and osteocytes/mature osteoblasts should be allowed to take control of collagen fibrillogenesis and mineralization under conditions that simulate the physiological biochemical and biomechanical environment of bone.

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**Conflict of interest statement**

Nothing declared.

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