Current Advances in Anisotropic Structures for Enhanced Osteogenesis

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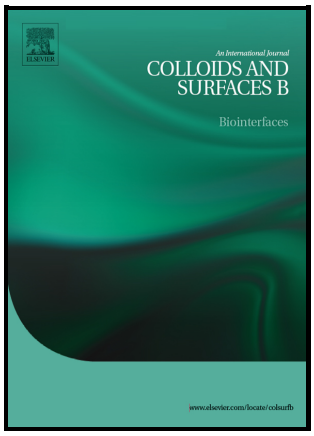

PII: S0927-7765(23)00444-7

DOI: https://doi.org/10.1016/j.colsurfb.2023.113566

Reference: COLSUB113566

To appear in: *Colloids and Surfaces B: Biointerfaces*

Received date: 26 August 2023
Revised date: 20 September 2023
Accepted date: 26 September 2023

Please cite this article as: Jishizhan Chen, Current Advances in Anisotropic Structures for Enhanced Osteogenesis, *Colloids and Surfaces B: Biointerfaces,* (2023) doi:https://doi.org/10.1016/j.colsurfb.2023.113566

Current Advances in Anisotropic Structures for Enhanced Osteogenesis

Jishizhan Chen[1,*]

[1] UCL Mechanical Engineering, University College London, WC1E 7JE, UK

* Correspondence: jishizhan.chen@ucl.ac.uk

Abstract

Bone defects are a challenge to healthcare systems, as the aging population experiences an increase in bone defects. Despite the development of biomaterials for bone fillers and scaffolds, there is still an unmet need for a bone-mimetic material. Cortical bone is highly anisotropic and displays a biological liquid crystalline (LC) arrangement, giving it exceptional mechanical properties and a distinctive microenvironment. However, the biofunctions, cell-tissue interactions, and molecular mechanisms of cortical bone anisotropic structure are not well understood. Incorporating anisotropic structures in bone-facilitated scaffolds has been recognised as essential for better outcomes. Various approaches have been used to create anisotropic micro/nanostructures, but biomimetic bone anisotropic structures are still in the early stages of development. Most scaffolds lack features at the nanoscale, and there is no comprehensive evaluation of molecular mechanisms or characterisation of calcium secretion. This manuscript provides a review of the latest development of anisotropic designs for osteogenesis and discusses current findings on cell-anisotropic structure interactions. It also emphasises the need for further research. Filling knowledge gaps will enable the fabrication of scaffolds for improved and more controllable bone regeneration.

Keywords: Anisotropic structures; Osteogenesis; Bone tissue engineering; Biomaterials; Signaling pathways

1. Introduction

Large bone defects are defined as those exceeding the critical size (2.5 cm) [1] that cannot self-recover without intervention. They are considered a major problem for clinicians and society. They are caused by various reasons, including but not limited to aging, genetics, trauma, severe diseases such as cancer, and infections. With an increase in global population aging, it is only reasonable to expect the cost of treating the number of bone defects to increase and become a huge burden on society. Approximately 2.2 million bone graft procedures per year are carried out worldwide[2]. In the United Kingdom (UK), there are approximately 850,000 new fractures seen each year. However, there are still 5-10% rates of nonunion of fractures, which adds additional costs for the National Health Service (NHS)[3]. The most commonly used conventional methods for reconstruction of large bone defects are vascularised fibula

autografts[4], the Ilizarov technique[5], and the Masquelet technique[6]. The vascularized fibula autograft provides a reliable source of vascularized bone graft, which promotes healing and can be used in various anatomical sites. It is suggested for defects of 5 to 12 cm. However, considerable donor site morbidity[7], longer operative time for microsurgery[8], and inadequate hypertrophy of the graft for the use of vascularized fibular autografts[9] are the limitations of this technique. The Ilizarov technique relies on distraction osteogenesis and is a leading option for defects of 2 to 10 cm. This minimally invasive approach offers precise control over bone regeneration. However, it requires an even longer duration of wearing a burdensome external fixation, resulting in an increased infection rate, joint stiffness, and negative influence on daily life quality[10]. The Masquelet technique[6] induces a biological membrane around the bone defect sites using a spacer, promoting formation of a vascularised bed at the first stage. After that, different cancellous bone graft materials without vessels can be implanted at the second stage. It is suitable for the administration of complicated bone defects, and it has been reported that defects of up to 25 cm can be regenerated[11]. On the other hand, the major disadvantages of this technique are the prolonged reconstruction period and multiple operations. Whether the aforementioned methods are used, a long and difficult process is inevitable. There also remains a critical issue that the final functional outcome is still unpredictable.

In recent decades, the rapid development of bone tissue engineering has brought new solutions. Since the word ‘tissue engineering’ was mentioned for the first time in 1987[12], it evolved into a multidisciplinary term of ‘regenerative engineering’ with the advances and convergence of biomaterial science[13], stem cells[14], induced pluripotent stem cells (iPSCs)[15], and developmental biology[16]. In general, engineered artificial bone appears in the form of scaffolds with or without cell laden [17, 18]. Although a range of biomaterials have been developed, including metallics[19-22], inorganics[23-25], organics[26-29], and composites[30-32], there are unsolved limitations such as insufficient biofunctionality, low mechanical properties, and cytotoxicity. Biomaterials that can fully mimic natural bone in structure, function, and mechanics have not yet been found. Nevertheless, the understanding of cell-material interactions and advances in micro- and nanofabrication are narrowing the gap. It is widely known that the microenvironment (also known as the ‘niche’) intensively affects bone cell behaviours throughout the entire cell life span, including attachment[33], proliferation[34], differentiation[35], secretion[36], apoptosis[37], and even cancer-related activities[38]. Apart from mimicking the chemical microenvironment by formulating bone-like components, the remarkable influence of the physical microenvironment is drawing increasing attention. An increasing number of researchers have realised that topographic or structural cues are as important as mechanical properties, especially in bone, an organ with anisotropic structures at multiple scales. In this review, we introduced the hierarchical structure of bone and the latest knowledge about the role of the physical microenvironment, specifically, anisotropic structures, in bone regeneration. Then, current strategies for fabricating anisotropic structures were reviewed (Figure 1), followed by signalling pathway analysis. This review provides a thorough retrospective assessment of the progress of anisotropic designs for bone regeneration and indicates future trends and higher requirements in bone biomimetic scaffolds.

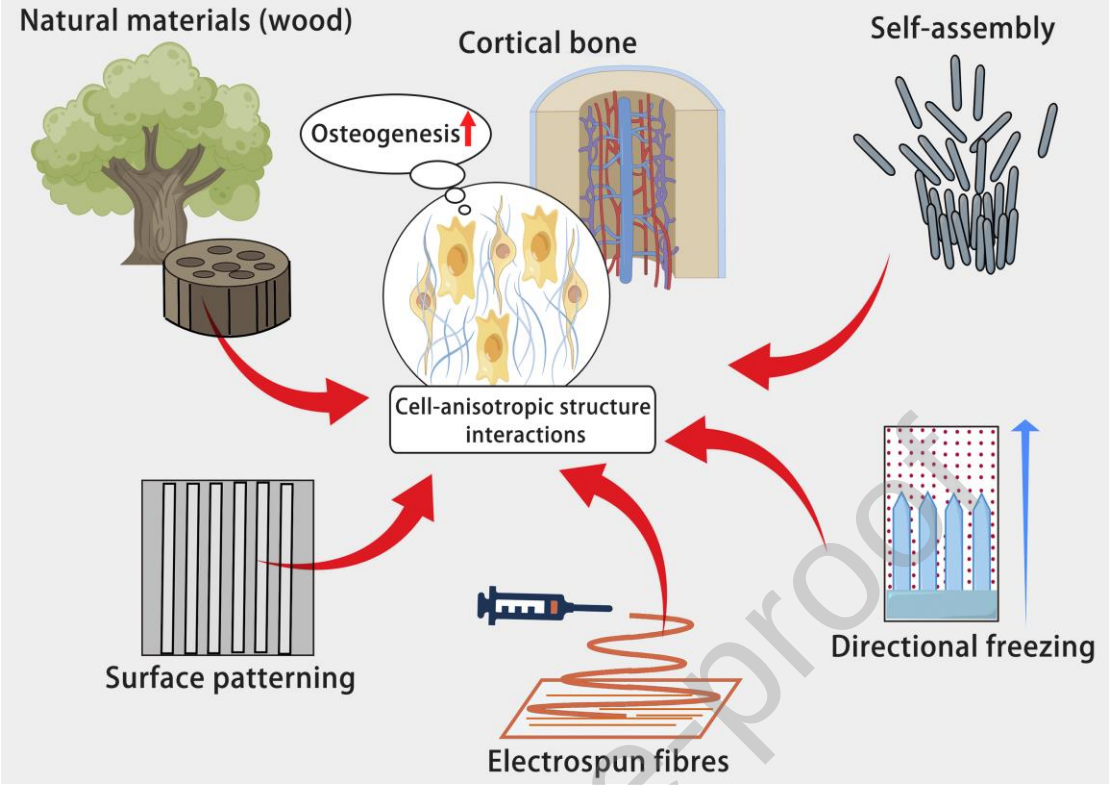

Figure 1. Current strategies for fabricating anisotropic structures for improving osteogenesis.

2. Anisotropic structure of cortical bone and the corresponding role

2.1 Anisotropic structure of cortical bone

Bone is a supporting structure as well as a storage site for minerals and blood cells. It is classified into three types: compact tissue, cancellous tissue, and subchondral tissue[39, 40]. Compact tissue is located at the outer covering and is known as cortical bone. It is highly mineralised and contributes to most of the mechanical strength of bone. Cancellous tissue is the inner sponge-like trabecular bone. Subchondral tissue represents the transient structure at the end of the bone and is covered by cartilage tissue. Human cortical bone orchestrates organic and inorganic phases at multiple size scales into ordered and anisotropic structures, strongly suggesting a liquid crystalline (LC) structure. The unique organisation of bone at the nano- and microscale gives it extraordinary stiffness, allowing it to resist much higher forces under both static (maintaining posture and bearing weight of the body) and dynamic (walking, running, lifting, etc.) conditions than most other tissues[41]. In natural bone, the organic component of bone is mainly composed of type I collagen molecules with a length of ~200 nm and a width of ~2 nm. In a typical cortical bone formation process, collagen molecules self-assemble into highly ordered fibrillar structures, leaving periodical minor gaps, in which HAp, the major inorganic component of bone, deposits and forms plate-shaped crystals 10–20 nm in length and 2–3 nm wide. There is evidence showing that this process is driven by the anisotropic piezoelectricity of collagen molecules[42]. Higher surface charges was found at the gap zone, resulting in an increased zeta-potential and hydraulic permeability to guide the calcium crystals infiltrate into the gap region. Then, the mineralised collagen molecules are packed into ~500 nm larger fibrils that aggregate into 1 – 10 μm collagen fibre bundles[43]. Dense lamellas compacted from the collagen fibres dispose in two alternating directions, form orthogonal

polywoods and further compose osteons, the elementary unit of cortical bone, with a size of 10–500 μm[44] (Figure 2a).

In 1906, Gebhardt first introduced a model of bone microstructure: compacted collagen fibres dispose in two alternating directions to form orthogonal polywoods. The lamellar structure of cortical bone was first defined as parallel collagen fibres altering 90° between adjacent lamellas[45]. In 1968, Ascenzi and Bonucci further described that the osteon is a highly ordered structure using polarising microscopy, and the orientation of collagen lamellae can be classified into three different types[46], two of which have been widely accepted as main models (Figure 2b). Although there are still no final conclusions about the bone model, it can be confirmed that cortical bone is a multilayer anisotropic structure with an altered arrangement between layers. In recent decades, liquid crystallinity in collagen systems has been reproduced at the molecular level in vitro. Collagen dissolved in acid solutions can gradually self-assemble and show a typical birefringence pattern of cholesteric liquid crystals at a high concentration of 80–120 mg/mL[47, 48]. Resembling bone microstructure to anisotropic liquid crystalline structures suggests that the self-assembly of mineralised collagen is associated with fluidity; thus, this must occur at the very beginning of extracellular matrix (ECM) secretion in the external space of cells. This information provides an inspiration for mimicking ordered deposition and self-assembly in vitro. Considering the coaxial lamellar structure of osten, the early-formed inner layer of parallel collagen fibres could be the clue inducing the cells' directional secretion and self-assembly to form later outer lamellae. Therefore, anisotropic biomaterials can promisingly be used as templates to manipulate cell secretion behavior in vitro.

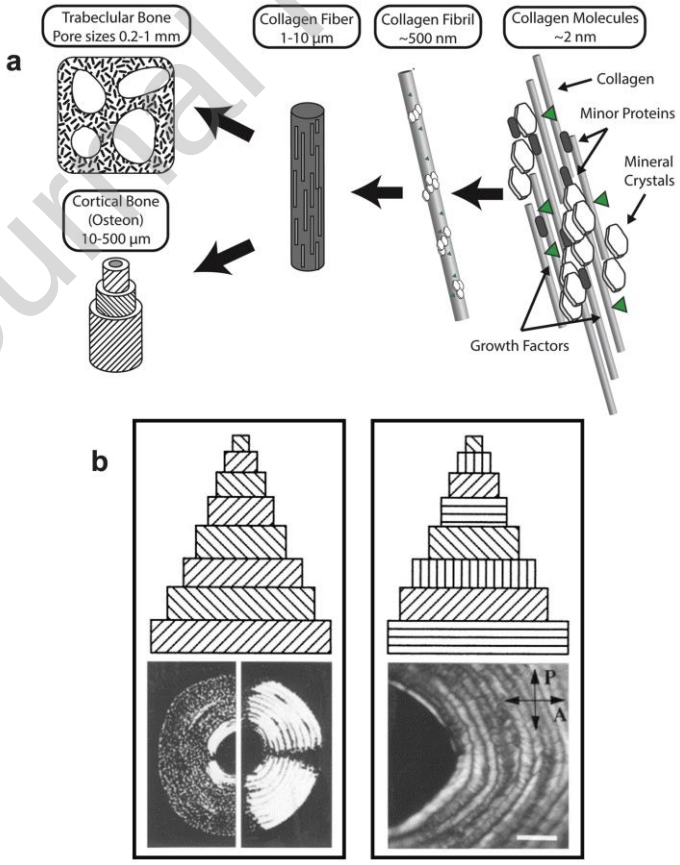

Figure 2. (a) Schematic of the ordered structure of bone at multiple scales. **(b)** Two main

models showing the orientation of adjacent lamellae in osteons. Left: orthogonal polywoods changing orientation periodically and forming alternate dark or bright rings; Right: intermediate-type osteons have adjacent lamellae successively changing from a small and constant angle (scale bar = 5 μm). Adapted from references[43, 49].

2.2 The mechanical and biological role of anisotropic structures in bone

Mineralised collagen fibrils (MCFs) are the fundamental unit of bone. The collagen and HAp are woven into an integrated anisotropic structure in the cortical bone, similar to a 'biphase' interpenetrating composite material where these two main components play different mechanical roles. Wang *et al*.[50] denatured collagen to various degrees using heat treatment at 37 – 200 °C without damaging the mineral phase of bone (changes in the mineral phases occurred only at temperatures above 400 °C[51]). The results show that the toughness of bone significantly decreased as the denaturation of collagen increased but had little effect on the stiffness of bone. The authors indicated that the loss of collagen network integrity, including cleaved peptides and/or damaged collagen-mineral bonding, may be one of the reasons for the reduced capacity to absorb energy to fracture. Organic components are the primary contributor to the toughness of bone[52]. In addition, we consider that the damaged alignment of collagen is also a factor resulting in decreased toughness. Since bone is anisotropic in nature, mechanical testing can help to verify this hypothesis. However, the authors only performed longitudinal mechanical tests, lacking data from the radial direction. More mechanical and optical data need to be collected before drawing conclusions. The unaffected mineral phases maintained the great stiffness of bone, which may be a result of the unchanged ordered arrangement of HAp. Martin *et al*.[53] also found that the anisotropic arrangement of collagen in equine cannon bone correlates with the modulus and strength of bone. Although the above studies have proven that the anisotropic and ordered arrangement of collagen/HAp is closely related to the mechanical properties of bone, their assessments were mostly carried out using collagen and HAp as a hybrid system. The outcomes could be comprehensive results of various factors. Since collagen and HAp are tightly integrated in bone, they bring huge challenges to polarising observation and mechanical evaluation of individual components. To better explain the individual characteristics of collagen and HAp in cortical bone, Novitskaya and colleagues[54] carried out comprehensive work investigating the mechanical influence of individual components in bovine cortical bone. In untreated (UT), demineralised (DM) and deproteinized (DP) cortical bones, all samples exhibit anisotropic mechanical behaviour. The highest stiffness was measured in the radial direction of UT bone due to the existence of a thin layer of circumferential lamellae (periosteal bone) that provides extra strength, while both DM and DP bones were stiffest in the longitudinal direction. Interestingly, the sum of strength of the DM and DP bones in all directions was not equal to that of the UT bone, suggesting a strong interaction between collagen and HAp, instead of simply mixing up or aligning these two components. The Young's modulus of DM bone was nearly 100 times lower than that of UT bone, providing evidence correlating with the aforementioned literature that minerals contribute most of the stiffness in bone. A high degree of alignment and intact collagen-HAp binding at multiple scales strengthen the bone through mechanisms of preventing the accumulation of microcracks via good energy dissipation. At the stage of elastic deformation, mineralised collagen fibrils undergo stretching and interfibrillar sliding[55]. With increasing loads, plastic

deformation leads to interfibrillar and intrafibrillar slipping and dissociation of collagen-HAp bonds[52, 55, 56]. Plastic deformation is the result of damage at the nanoscale. All these incidents are related to the damping mechanism of collagen fibril viscoelasticity, which promotes energy dissipation and postpones failure[56]. Similarly, Jäger and Fratzl[57] also indicated that the disordered arrangement of collagen and HAp can impede load transmission. The preferred orientation of MCFs is regarded as an adaptation of long-term external mechanical loads. For example, the anterior quadrant of the murine femur cortical bone withstanding stronger tension contains a greater proportion of longitudinal MCFs compared to the posterior quadrant bearing stronger compression[58]. In correlation, an in vivo study on mice shows that aging and disuse exacerbates the disorder of MCFs and impairs mechanical properties[59]. Similarly, disease-caused damage to collagen/HAp orientation can contribute to increased strain and reduced mechanical properties. A mouse osteoporosis model revealed more heterogeneous collagen/HAp orientation of the L5 vertebral cortex, as evidenced by the observation of more random birefringence defect domains on the model by polarisingpolarizingpolarizing microscopy, along with decreased Young's modulus compared to the control group[60]. In osteoarthritis (OA) models[61], collagen fibrils of early-stage grade I OA were still in an ordered liquid crystalline pattern with the c-axis of HAp parallel to the long axis of the fibrils. In comparison, severe grade IV OA formed a random arrangement of collagen fibrils accompanied by shape changes in HAp. The nonparallel arrangement of collagen and HAp in developed OA leads to abnormal load transmission and difficulties in stress dissipation, eventually causing fragility and weakening resistance to elastic deformation at the macroscale. In summary, MCFs serve as the nanoscale unit of bone, and the interaction between collagen and HAp through anisotropic and ordered arrangement plays a crucial role in the mechanical properties of bone. The mineral phase contributes significantly to the stiffness of bone, while the ordered arrangement and intact collagen-HAp bonding enhance bone strength and facilitate effective energy dissipation by preventing the accumulation of microcracks. The denaturation and failure of collagen-HAp bonding and damage of anisotropy can lead to a reduced capacity of the bone to absorb energy.

The biological functions of anisotropic arrangement intrigued researchers. Cells are capable of sensing nanoscale cues and tuning their behaviors accordingly[62]. A computational modelling analysis suggests that the anisotropic microstructure may imply a preferential direction for cell invasion, deciding the spatial and directional distribution of newly formed bone[63]. Among all biomaterials, type I collagen and HAp are the most popular, as they are natural components of bone. These two biomaterials both have the capability to be organised into anisotropic structures under specific conditions, which is the unique arrangement in natural cortical bone. Some pioneering work investigated the influence of anisotropic collagen on a few types of cells. Normally, aligned collagen is prepared by shearing force. The seeded endothelial cells showed elongated morphology along the orientation of aligned collagen fibrils and enhanced proliferation, migration, and angiogenesis. In this process, integrin-mediated focal adhesions function as pivotal regulators of the cytoskeleton[64, 65]. The integrins α1 and α2 strongly interact with type I collagen[66]. However, these studies only looked into a few protein markers and genes involved in topography-cell interactions based on experience, which is incapable of unveiling the full picture of the cellular response to anisotropic arrangement. Nakayama and

colleagues[67] fabricated HAp/poly(acrylic acid) (PAA) nanorods into aligned films and used them as a photodynamic therapy platform for HeLa cells. This work preliminarily characterised the alignment of cells on orientated LC films and the treatment efficacy, but further mechanisms have not been discussed. Minami *et al*.[68] reported that aligned LC fullerene whiskers enabled concurrent control of C2C12 over cell elongation, alignment, and differentiation to muscle cells. Similarly, this work only focuses on characterising proteins and genes related to myogenesis and lacks in-depth mechanistic analysis of how cells respond to anisotropic arrangement. Previous studies have done limited work to reveal the mechanisms of anisotropically arranged collagen and HAp, especially for osteogenesis. A few reports, such as Wingender *et al*.[69] found that HAp nanocrystals were embedded within dense cholesteric LC collagen scaffolds during in situ mineralisation and that the preferential c-axis of HAp was aligned with the collagen fibril axis. However, the utilised LC collagen was only locally orientated, the effect of large-scale alignment has not been assessed, and the dense composites became an obstacle in observation. They emphasised the importance of the bone-like nanostructure in guiding cell behaviours, but no in vitro experiment has been carried out. Another study[70] using long-range ordered self-assembled collagen-like peptide amphiphile (CLPA) induced directional growth of MC3T3-E1 cells. Then, precipitation of calcium phosphate on the collagen-mimetic patterns was performed, but no lattice information of the minerals was reported. There remains a knowledge gap in the roles of anisotropic arrangement in osteogenesis. Notably, cell polarisation (elongation and orientation) appears to be a significant incident during differentiation and development into fully functioning cells. Inspired by the natural anisotropic structure of bone and previous reports, we believe that the structure is a critical clue but has long been neglected in investigating the process of bone formation.

3. Micro/ nanoscale anisotropic structures for osteogenesis

In bone regeneration and engineering, scaffold and stem cell combined strategies and artificial bone have become major emphases. Researchers have developed various materials, such as alloys[71] and natural or synthetic polymers[26, 72, 73], for in vitro evaluation or in vivo implantation. Some researchers have recognised the importance of incorporating anisotropic structures into scaffold fabrication to enhance mechanical stiffness and provide topographical cues for osteogenesis[74-76]. Fabricating anisotropic structures at the microscale is the strategy mostly adopted by researchers because it provides the best balance between osteogenic efficacy and difficulty in fabrication. In comparison, the characteristics of nanoscale materials can vary significantly from those of micro- or large-scale materials in terms of mechanical properties[77], optical properties[78], melting point[79], electrical conductivity[80], magnetic properties[81], and chemical[82] and biological reactivities[83, 84]. The large fraction of surface atoms, large surface energy, spatial confinement and reduced imperfections can contribute to this phenomenon[85]. Although microscale anisotropic structures have shown improved osteogenesis, cells can recognise nanoscale arrangements and may display different interactions compared to microscale arrangements[86, 87]. The biofunctions of nanoscale anisotropic structures are worth investigating. Here, we listed popular techniques that can fabricate anisotropic structures for the purpose of mimicking that of bone and discussed their advantages and limitations (summarised in Table 1).

3.1 Natural anisotropic materials

Nature provides a variety of ready-to-use materials with aligned porous structures. Wood is a fantastic material similar to bone, with modulus (hundreds MPa) falls within the range of human trabecular bone (between 10 and 3,000 MPa)[88]. It is mainly composed of lignin, cellulose, and hemicellulose. A broad range of woods was used for fabricating scaffolds and they have a highly aligned and interconnected network, as well as extraordinary mechanical strength. Wood is rich in sources and has relatively lower expenses compared to synthesised materials, making it an option for bone tissue engineering. Figure 3a and 3b show the types of natural woods and delignification processes used to remove unwanted woody components. Inspired by the hierarchical anisotropic structure of natural wood, Wang *et al.*[89] developed a biomimetic scaffold by impregnating hydrogels in a delignified wood template, followed by strengthening the composite by in situ mineralisation of HAp nanocrystals. The composite showed anisotropic mechanical properties and reached a compressive strength of 39.5 ± 0.9 MPa and an elastic modulus of 670 ± 11 MPa along the cellulose fibril growth directions, comparable to human trabecular bone. The in vitro experiment displayed enhanced osteogenic gene and biomarker expression, while the in vivo experiment confirmed new bone tissue ingrowth into and yielded good osteointegration with the scaffold. Introducing oriented wood texture as a skeleton followed by in situ mineralisation is a strategy for load-bearing bone repair. Liu *et al.*[90] fabricated delignified wood infiltrated with PCL and in situ deposited HAp. The composite showed anisotropic mechanical properties in the radial direction (420 MPa) and longitudinal direction (20 MPa). It has good biocompatibility and induces better cell migration, alignment, proliferation, and osteogenic differentiation than the control (Figure 3c – 3e). Similarly, Chen *et al.*[91] reported chitosan quaternary ammonium salt (CQS) and dimethyloxalylglycine (DMOG) treated wood-based anisotropic scaffolds. CQS enhanced the antibacterial ability, while DMOG significantly improved osteogenesis of human bone marrow-derived stem cells (hBMSCs) and angiogenesis of human umbilical vein endothelial cells (HUVECs). They also identified that the treated wood scaffolds promoted osteogenic differentiation of the hBMSCs via Yes-associated protein (YAP)/transcriptional co-activator with PDZ binding motif signaling pathway. Hu *et al.*[92] developed highly strong delignified white wood/regenerated silk fibroin hydrogel scaffolds integrated with black phosphorus quantum dots (BPQDs) encapsulated by poly (lactic-co-glycolic acid) (PLGA), which can reach 300 MPa at longitudinal direction and potentially function in both bone regeneration and ablation of bone metastasis. Another research group designed delignified white wood scaffolds filled with polyvinyl alcohol (PVA) hydrogel loaded with curcumin (Cur) and phytic acid (PA) for enhanced antibacterial, anti-inflammatory, and osteogenic activities[93]. Different properties (stiffness, porosity, etc.) in different types of wood make it an interesting topic for comparing and selecting the most suitable types for bone tissue engineering. Simultaneously, it is suggested to explore more modification techniques can further improved the wood-based scaffolds with various biofunctions. The drawbacks of using delignified wood as scaffolds are obvious. The delignification process can be time-consuming and complex. Although wood-based scaffolds can reach a compression modulus of hundreds of MPa, their strength is still much lower than the GPa of natural cortical bone. This limits its long-term utilisation in load-bearing sites. Otherwise, delayed union or nonunion of bone fracture may occur due to

mismatched mechanical strength[94]. Moreover, the microstructure of wood depends on its nature, so it is difficult to finely control or modify. This character also makes it challenging in terms of scalability and reproducibility, as the properties of natural materials can vary significantly depending on the source and processing method. Due to these significant disadvantages, wood poorly fits in the current trend of chasing precision manufacturing.

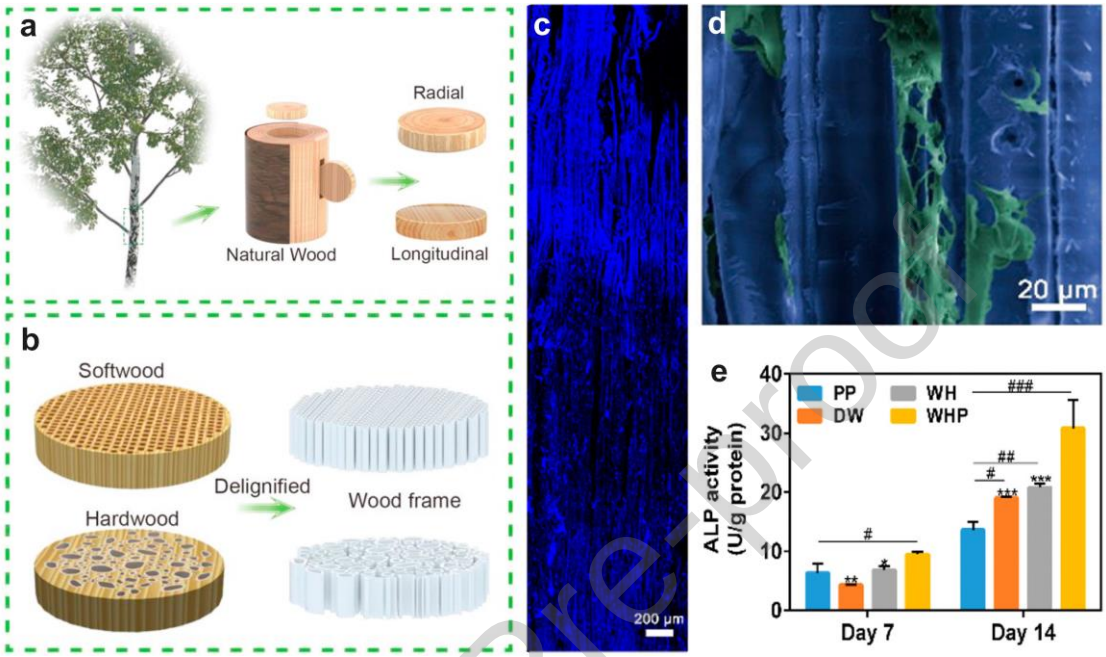

Figure 3. (a) Schematic of two main types of wood frames, radial and longitudinal, that are generally used for processing; **(b)** Process of delignification. The delignified wood can be used as frames for polymer infiltration. Adapted from reference[95]. **(c)** The longitudinal view of immunofluorescence shows that the wood-hydroxyapatite-polycaprolactone (WHP) composite facilitated even distribution of MC3T3-E1 cells (blue). Scale bar = 200 μm. **(d)** The longitudinal false-coloured SEM image shows that the MC3T3-E1 cells (green) were elongated and aligned along the direction of the wood channels (blue). Scale bar = 20 μm. **(e)** A significantly higher ALP activity was measured from the MC3T3-E1 cells cultured on WHP scaffolds. PP: pure porous PCL; DW: delignified wood; WH: wood-hydroxyapatite; WHP: wood-hydroxyapatite-polycaprolactone. Adapted from reference[90], Copyright 2020 American Chemical Society.

3.2 Surface patterning with parallel grooves

Modification of surface topography is one of the most straightforward and cost-effective methods of creating anisotropic structures. Parallel grooves are actually comprised of three crucial parameters: ridge width, gap width, and gap depth (or ridge height). Their combinations significantly affect the cell-material interaction. Normally, embossing and moulding (or microimprinting) are two popular techniques. Apart from the aligning effect in cells and extracellular matrix, which can enhance osteogenic differentiation, it also shows the potential to improve the integration between the scaffold and the surrounding tissue. Nadeem *et al*.[96] demonstrated that calcium phosphate/gelatin composite scaffolds with surface micropatterns of parallel grooves could upregulate the expression of the osteogenic biomarkers osteopontin (OPN) and osteocalcin (OCN) in human osteoprogenitor cells (hOPCs). The in vivo experiment showed that both the 50 μm and 40 μm groove patterned groups had better bone-scaffold

integration and more newly formed bone than the nonpatterned control group. In another study, parallel grooves on silica films guided human osteoblast-like cells into an aligned and elongated morphology, with enhanced ALP activity on a 45 μm spacing pattern[97]. The optimal groove spacing for osteogenesis seems to be inconsistent on different materials or is also related to the cell type. For example, MC3T3-E1 osteoblasts on patterned titanium were found to have improved cell length, elongation, and alignment, as well as increased expression of osteogenic genes and ALP activity, by increasing the groove width from 3 – 7 μm. The maximum stimulating effect peaked at a specific groove pattern with ridge width = 3 μm, groove width = 7 μm, and groove depth = 2 μm[98]. Interestingly, changes in groove depth from nanoscale (35 nm) to microscale (2 μm) on poly(lactic-co-glycolic acid) (PLGA) substrates have no facilitating effect on osteogenesis[99]. The limitations of this approach are challenges in confirming the most effective pattern for a specific material and a cell type, which could be time-consuming work and not repeatable in other materials and cell types. Furthermore, the technique is more suitable for creating patterns on (two-dimensional) 2D planes. Creating patterns in 3D significantly increases the complexity in fabrication. Long-term efficacy is also doubtable, as cell secretions could fill in the pattern and turn it to a flat surface after a period. Currently, no study has assessed the long-term efficacy of groove patterns. Finally, the grooves may not be suitable for load-bearing applications, as this pattern may weaken the mechanical properties of scaffolds. Surface patterning modification with parallel grooves is a promising method for bone regeneration scaffolds. It is convenient for investigating molecular mechanisms in vitro on a 2D plane. However, further studies are needed to optimise the pattern and evaluate its long-term effectiveness both in vitro and in vivo.

3.3 Directional freezing

Directional freezing is an ingenious method for obtaining aligned structures without introducing additional chemicals. Its versatility allows it to produce a wide range of intricate shapes of either porous three-dimensional structures or two-dimensional patterns on surfaces, including nanocomposites composed of polymers and inorganic materials, networks of aligned gold microwires, porous composite microfibers, and biaxially aligned composite networks[100]. Bioactive components can also be well preserved under low temperature. Figure 4a illustrates the process of directional freezing. Generally, the control of directional freezing relies on differences in temperature at two points of the subjects. The ice crystals then directionally grow from the low-temperature point towards the relatively high-temperature point, forming interval micro ice chambers. After freeze drying, the ice crystals sublimate and leave aligned interconnecting hollow channels[101, 102]. Some anisotropic structures have been investigated for their potential in enhancing osteogenesis. In 2006. Deville *et al*.[103] developed directionally frozen porous HAp scaffolds (Figure 4b and 4c) with high compressive strength up to 145 MPa for 47% porosity and 65 MPa for 56% porosity. In that study, the authors discussed parameters influencing the properties of the scaffolds. However, no biocompatibility or degradation tests were performed. The performance of the anisotropic scaffolds made from HAp slurry needs further testing. Maleki *et al*.[104] developed a hybrid scaffold made of silica and silk fibroin bioaerogel for bone regeneration (Figure 4d). The scaffold had a honeycomb micromorphology with a multiscale aligned porous structure created using directional freezing (Figure 4e). The authors evaluated the mechanical properties and biocompatibility of the

scaffold in vitro and in vivo and found that it had excellent mechanical strength, supported cell growth and osteogenic differentiation, and promoted new bone formation in a rat model. The scaffold also exhibited good biodegradability and bioactivity. It is suggested that the anisotropic silica-silk fibroin bioaerogel scaffold could be a promising candidate for bone tissue engineering. Su *et al*.[105] prepared a bilayered gradient poly(vinyl alcohol) (PVA)/HAp composite hydrogel by directional freezing. PVA hydrogels with aligned structures were first fabricated using directional freezing, followed by coating with HAp using electrophoresis. The composite reached up to 0.27 MPa tensile modulus and 0.51 MPa compressive modulus and supported the attachment of osteoblasts. Silk fibroin/nano-HAp/graphene oxide composite scaffolds with directional channels were fabricated by Wang and colleagues[106] using directional freezing. It was demonstrated that the scaffolds were beneficial to the adhesion and proliferation of BMSCs, upregulated osteogenic genes in vitro, and had better bone integration capabilities in vivo. Recently, some researchers have realised using anisotropic structures to exclude interference from nonosteogenic cells and fibrous tissues during bone regeneration[107]. By adjusting the temperature gradient, the direction of ice crystal formation can be controlled. The directionally frozen scaffolds comprised chitosan and porcine cortical bone-derived HAp nanoparticles. Scaffolds with either radial or axial pores were fabricated. Both in vitro and in vivo experiments confirmed that the scaffolds were highly biocompatible and effective in promoting biological functions. The scaffolds had pore channels that could guide cell infiltration, while the absence of open pores on the channel walls prevented surrounding cells and tissues from invading the scaffolds. Moreover, further in vivo tests showed that the radially oriented porous structure had the potential to promote osteogenesis while preventing interference from nonosteogenic cells and fibrous tissue in lacunar bone defect repair compared to the axially oriented porous scaffold. Similarly, in another report, directional migration of bone-related cells from the host tissue toward the center of the defect was found on radially frozen mineralised collagen scaffolds with nanosilicon incorporated[108]. Periosteum-diaphysis biomimetic directional silk fibroin scaffolds also prevented the growth of fibrous tissues in rabbit bone defect models and thus reduced the occurrence of nonunion[109]. Interestingly, directional freezing can also be applied to piezoelectric materials, providing multiple cues to induce osteogenesis. Tang's group[110] fabricated 1-3-type BaTiO3/PMMA biopiezoelectric composites. These scaffolds combine both topographical and electrical stimuli to osteoblasts, guiding their ingrowth following the layer direction. However, the secretion or signaling pathways of the cells were not evaluated in this study. There are several main drawbacks in directional freezing. Contradictions between polymerisation and freezing are inevitable. Polymerisation is a critical step for maintaining a stable shape of polymers in wet conditions. However, crosslinked structures may interfere with ice crystal growth, creating less aligned channels. On the other hand, although freezing the solution directly before polymerisation gives more aligned microstructures and better controllability, crosslinking cannot be performed on freeze-dried scaffolds in some situations. For example, to stabilise a freeze-dried collagen scaffold without crosslinking, immersing the scaffold in a crosslinker leads to rapid dissolution and loss of shape before being crosslinked. The freeze-drying process also consumes much energy, as the vacuum pump and freezer need to be on working for at least 24 to 48 hr. Directional freezing is a promising method for producing anisotropic scaffolds for bone regeneration, with advantages such as versatility, excellent mechanical strength,

biocompatibility, and biodegradability. Further research is needed to optimise the fabrication process, evaluate the efficacy of anisotropic structures in vivo, and determine the long-term stability and efficacy of directionally frozen scaffolds.

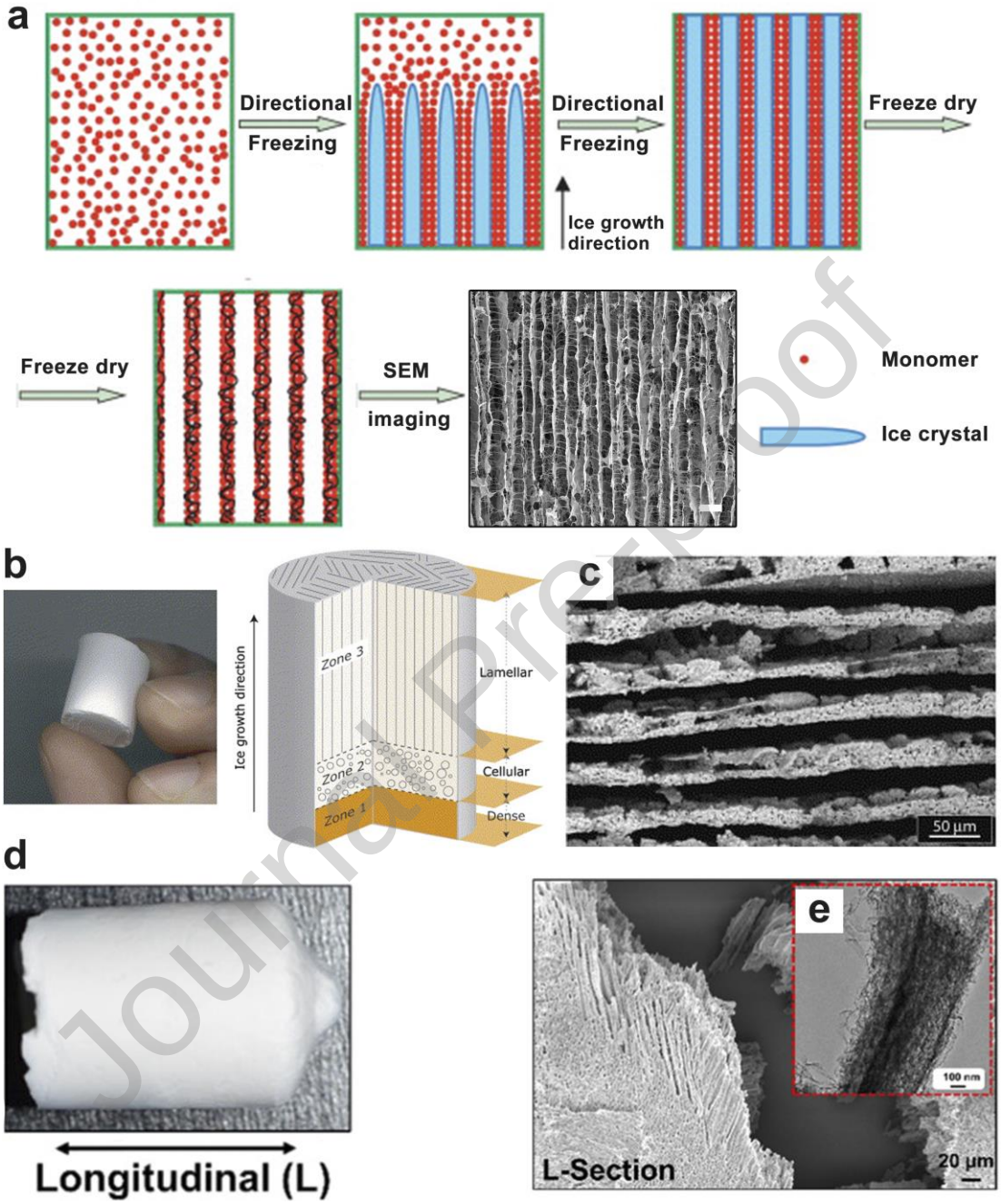

Figure 4. (a) Schematic of temperature gradient-driven directional freezing. Ice crystals directionally grow from the low-temperature point towards the high-temperature point. After sublimation, hollow structures form in the place that was ice. Sacle bar = 100 µm. Adapted from references[111, 112], Copyright 2015 American Chemical Society (reference[112]). **(b)** A directionally frozen HAp scaffold (left) and illustration of the hierarchical microstructure of the porosity (right). Generally, three distinctive zones can be found in directionally frozen scaffolds. **(c)** SEM image of the longitudinal cross-section parallel to the growth direction of ice crystals. Adapted from reference [103]. (d) Gross view of silica and silk fibroin hybrid bioaerogel after directional freezing, and (e) SEM longitudinal view shows aligned micro channels. Adapted

from reference [104], Copyright 2019 American Chemical Society.

3.4 Micro self-assembly

Self-assembly is a process in which individual components with a high length-to-diameter ratio can spontaneously form an organised structure under specific conditions. Using self-assembly, particles can be organised into highly aligned arrangements at the nanoscale. For instance, by adding HAp rigid phases into alginate solution, HAp influenced the phase transition during the fabrication of alginate/HAp sponges, forming macrodomains with anisotropically aligned pores. The sponges effectively supported the attachment and proliferation of human dental pulp mesenchymal stem cells (hDPMSCs) in vitro and the development of newly formed mineralised matrix at the site of cranial defects in in vivo models[113]. Lin's group[114] displayed a noncovalent assembly mediating preorientation-assisted strategy to fabricate highly anisotropic chitin/nanoscale 2D material (molybdenum disulfide and brushite, for example) composite hydrogels. The long-range aligned structure was obtained through mechanical deformation, such as shear force. In the reported process, the 2D materials were temporarily oriented under shear force, followed by polymerisation to achieve a permanent anisotropic microstructure. The orientation process significantly increased the strength and Young's modulus of the composites. In vitro culture of rat BMSCs on the hydrogels and implantation of the hydrogels into rat calvarial defect models both demonstrated improved cell migration and osteogenesis. Similarly, a biomimetic composite consisting of strontium (Sr)/copper (Cu)-doped 1D HAp and poly(dl-lactide) (PDLA) was developed[115]. In the composite, the self-assembly effect drove the c-axis of the HAp crystals to align with the PDLA polymer chains. Then, large-scale alignment is achieved via shear force when extruded from the nozzle. The anisotropic composite allowed sustained release of Sr and Cu ions, supported attachment and proliferation, enhanced ALP activity, and induced secretion of the anisotropic collagen fibre matrix of hMSCs. Mredha *et al*.[116] demonstrated fish swim bladder collagen (SBC)/poly(N,N'-dimethylacrylamide) (PDMAAm) double-network hydrogels. Taking advantage of the shear- and diffusion-induced collagen fibril orientation, the SBC self-assembled into an aligned and concentric organisation, was then enhanced by polymerisation of the PDMAAm network, and was coated with HAp on the surface. The composite reached a Young's modulus of 0.45 – 0.8 MPa, which is comparable to that of natural cartilage. In vivo experiments showed that the hydrogel has excellent bonding ability with bone. Ma *et al*.[117] reported anisotropic protein organofibers with extraordinary mechanical strength that could improve osteogenesis. The fibres were made of genetically engineered proteins that self-assembled into supramolecular structures with controlled orientations. BMSCs were found to have enhanced expression of the osteogenic biomarker RUNX2 on the fibres, which demonstrated their potential as a regenerative matrix in bone tissue engineering. In conclusion, self-assembling structures may represent the closest to the process of bone formation. The use of self-assembly allows for precise control over the size, shape, and orientation of the scaffold. However, a main obstacle facing the self-assembly technique is the difficulties and complexity in fabrication, making it currently difficult to scale up for clinical applications. The use of self-assembly may result in poor control over the final structure, leading to variations in scaffold properties. Additionally, there may be limited types of materials that have self-assembling capability, and currently, no self-assembled materials can reach mechanical strength comparable to that of natural bone.

3.5 Electrospun nanofibres

Electrospun fibres have intrinsic advantages in anisotropic structure and processability. Fibres can be easily spun into an aligned nanopattern and form films, supporting cell attachment, proliferation, and differentiation. A type of PCL/gelatine-aligned fibre was designed to enhance alveolar bone regeneration. It effectively stimulates macrophages to polarise towards the M2 phenotype, promotes bone immunoregulation and subsequently promotes the recruitment and osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs)[118]. However, the fibres are less feasible to fabricate into stiff and thick scaffolds with comparable mechanical properties to bones. PCL scaffolds coated with nanoscale PCL-aligned electrospun fibres (Figure 5a – 5c) explored the possibility of incorporating dual-scale topographical cues to influence human adipose-derived stem cell (hADSC) behaviour[119]. The cells were stretched and elongated along the fibres (Figure 5d), with improved overall osteogenic biomarker (COL-1, ALP, and OCN) expression. Nevertheless, there was no significant difference between the fibre-coated and naked PCL scaffolds. This could be a result of insufficient bioactivity of PCL substrates. Similarly, another group tried to combine electrospinning and photolithography techniques to create hybrid scaffolds with both nano- and microtopography (Figure 5e)[120]. Isotropic fibres (SU-8) and parallel grooves (on silicon wafer) mimicked the multilamellar helicoidal plywood model of bone, which promoted the proliferation, alignment, and osteogenic differentiation of hADSCs (Figure 5f). Fan *et al.*[121] developed anisotropic silk nanofiber-guided cell migration, along with enhanced angiogenesis by deferoxamine release, and the osteogenesis of hBMSCs was improved on the nanofibers. Osteoblasts were found with elongated and aligned morphology and enhanced mineralisation with c-axis orientation. Although some limitations constrain the application of electrospun fibres in bone tissue engineering, bioactive coating on the surface of PCL fibres and utilising fibres as a secondary additive to increase the topographical complexity of bulk scaffolds are promising approaches for enhancing osteogenesis. Overall, electrospun fibres show potential as bone regeneration scaffolds, but they are difficult to use as independent scaffolds in bones due to their inability to withstand loads and difficulties in forming enough spatial volume. Normally, electrospun nanofibers serve as surface modifications for solid bases. Further research is needed to address the limitations associated with their use and to optimise their effectiveness in promoting bone formation.

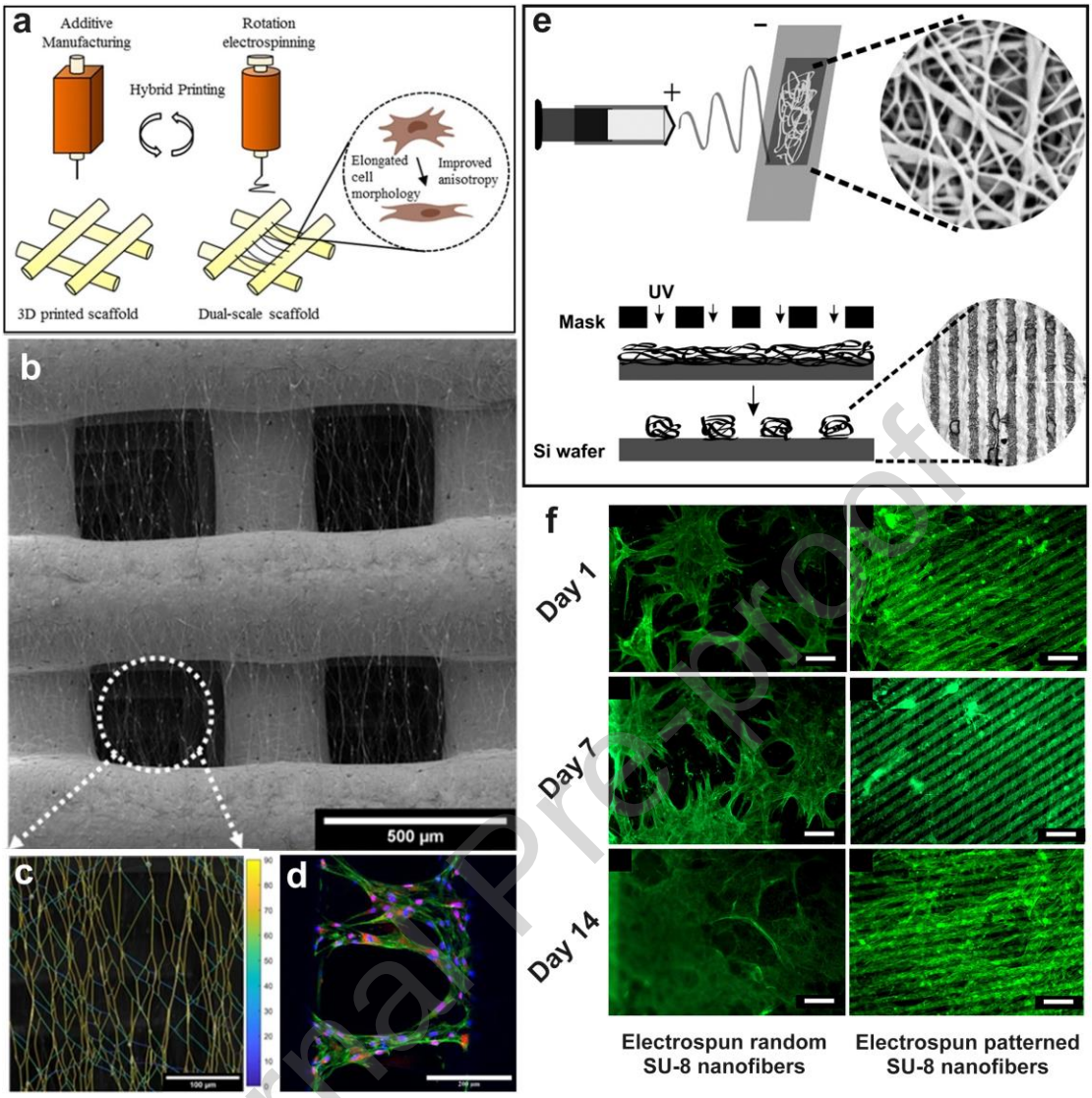

Figure 5. (a) Schematics of a hybrid surface modification process. Aligned PCL nanofibers were electrospun onto the 3D-printed PCL scaffolds. **(b)** SEM image of a nanofiber-modified scaffold (scale bar = 500 µm) and **(c)** magnification view of the anisotropically aligned nanofibers (color bar shows orientation, unit: °; scale bar = 100 µm). **(d)** hADSCs cultured on the hybrid anisotropic scaffolds displayed elongation and increased cell alignment anisotropy. Blue: nuclei, green: F-actin, red: collagen type I. Scale bar = 200 µm. Adapted from reference [119]. **(e)** Schematics of another study that fabricated electrospun SU-8 nanofibers with an anisotropic micropattern (20 µm ridges and grooves) using UV treatment. **(f)** Elongated and aligned hMSC morphology along the orientation of patterned nanofibers was observed on day 14 compared to the random morphology in the control group with random SU-8 nanofibers. Green: F-actin. All images scale bar = 100 µm. Adapted from reference [120].

3.6 Liquid crystals (nano self-assembly)

Liquid crystals are a typical form of nanoscale self-assembled structures. A liquid crystal is defined as a substance with a state of matter between liquid and solid. This mesophase may flow like a liquid, but its molecules are oriented in a crystal-like arrangement (Figure 6a). In 1888, Friedrich Reinitzer, an Austrian botanist, described his discovery of liquid crystals for

the first time and started the saga of liquid crystals in the following hundred years[122]. He extracted cholesteryl benzoate from a carrot's root and amazingly found that it had two 'melting points'. The solid cholesteryl benzoate structure transformed into a turbid liquid (later identified as a liquid crystal) at 145.5 °C and became transparent at 178.5 °C. This demonstrates that cholesteryl benzoate has three different phases: solid, liquid crystal, and liquid. Otto Lehmann (1904) later identified more organic compounds with properties similar to Friedrich's findings and originated the term 'liquid crystal'[123]. Basically, there are three types of liquid crystals: nematic, smectic, and cholesteric, classified by molecular arrangement (Figure 6a). The molecules in nematic liquid crystals are aligned parallelly, while in smectic liquid crystals, they not only show molecular alignment but also form a layered structure. In cholesteric liquid crystals, the arrangement is similar to that of smectic liquid crystals, but the orientation of molecules varies between layers and forms a helical structure.

Biological liquid crystals were first recognised by Lehmann in 1922. He realised that the liquid crystalline structure may play an important role in many forms of life[123], and his proposition has been validated and extended over the years. Long-range ordered arrangements can be observed not only in a liquid crystalline phase but also in a solidified phase from LC. All biological liquid crystals with anisotropic properties either are, or once were, in the lyotropic phase. LC organs have become one of the main structures in numerous forms of life due to their unique characteristics — mild biological condition-driven self-assembly, hierarchical units for signal transduction and stimulus response, and irreplaceable biofunctions. Over eons of evolution, nature has crafted numerous nanoscale biological structures with specific purposes. Biological liquid crystals are common examples that endow different forms of life with unique biofunctions. For example, the most eye-catching example of biological liquid crystals is the iridescent colour of beetle cuticles, which was first observed by Michelson in 1911[124] and was later interpreted as the circularly polarised reflection of beetle cuticles serving as communication channels for mate signalling and mate choice[125, 126]. In eukaryotes, cilia or flagella are critical for movement[127]. A special pattern known as the '9 + 2 array'[128] can be found in many types of animal or plant cells with cilia and flagella, from bacteria to the sperm tails. This obvious anisotropic and ordered structure with a high length-to-diameter (L/D) ratio strongly suggests an LC self-assembly process when the cilia and flagella are formed. Natural silk can be roughly described as multiblock copolymers reinforced by β-sheet domains of a relatively elastic protein matrix. It self-assembles and forms nematic liquid crystals with extraordinary elasticity[129, 130]. Human cortical bone has nanoscale LC structures; hence, nanoscale self-assembly may represent the closest process for mimicking bone formation and may have the highest potential for facilitating osteogenesis.

Rod-like stiff particles can be manipulated into LC status. Aligned halloysite nanotubes on solid substrates were fabricated by Zhao *et al.*[131] via a shearing method with brush assistance (Figure 6b). The seeded hBMSCs were well aligned along the clay nanotube orientation (Figure 6c) and exhibited promoted ALP activity and osteogenic gene expression (Figure 6d). In another work, researchers have fabricated biomineralised chitin nanowhisker (CHW)/ poly(ethylene glycol) diacrylate (PEGDA) hydrogels with bone-like chiral nematic LC state (Figure 6e)[132]. It was found that the CHW/PEGDA LC hydrogels induced higher protein

absorption and improved osteogenic differention of rat BMSCs, compared to CHW/PEGDA non-LC hydrogels. However, the authors haven't provide further evidence explaining the mechanisms. Apart from stiff particles, soft collagen nanofibrils can also self-assemble into anisotropic cholesteric liquid crystals by molecular crowding, triggered when the concentration reaches a critical point (normally over 80 – 100 mg/ml[133]). Wingender and colleagues [69] used the polymer-induced liquid-precursor (PILP) process to fabricate bone-like 'lamellar' structures. Concentrated collagen LC scaffolds were mineralised to achieve a high degree of intrafibrillar mineralisation. The mineralised structures showed anisotropic diffraction arcs of HAp crystals and [001] oriented along the c-axis of the fibrils (Figure 6f and 6g). This work demonstrates that nanoscale self-assembly can guide the formation of oriented minerals, but cell-material interactions have not been assessed. In another work, Wu *et al.* [134] developed anisotropic LC octyl hydroxypropyl cellulose ester (OPC)/polyurethane (PU) soft substrates to study the soft elastic response resembling the physical microenvironment of the stem cell niche. They found that the addition of varying liquid crystal concentrations had great effects on the surface morphology and elastic modulus of the substrates. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) were intensively affected by different doping rates of LC OPC, and their ALP activity and calcium deposition were significantly improved on substrates with 10% and 30% w/w OPC. In summary, LC structures can be compatible with the bone microenvironment and may serve as guides in cell fate commitment. The architecture of LC interfaces can be considered a bioinspired approach to impact cellular behaviours. However, current methods have difficulty realising fine control of LC particles/fibres and are unable to form bulk scaffolds. There is also a knowledge gap between the effect of general micro/nano patterns and LC nano patterns. The applications of liquid crystals are currently confined to coating or surface modification. There is an urgent need to develop reliable approaches for controlling liquid crystals at a large scale and comprehensively compare their biofunctions with those of other materials.

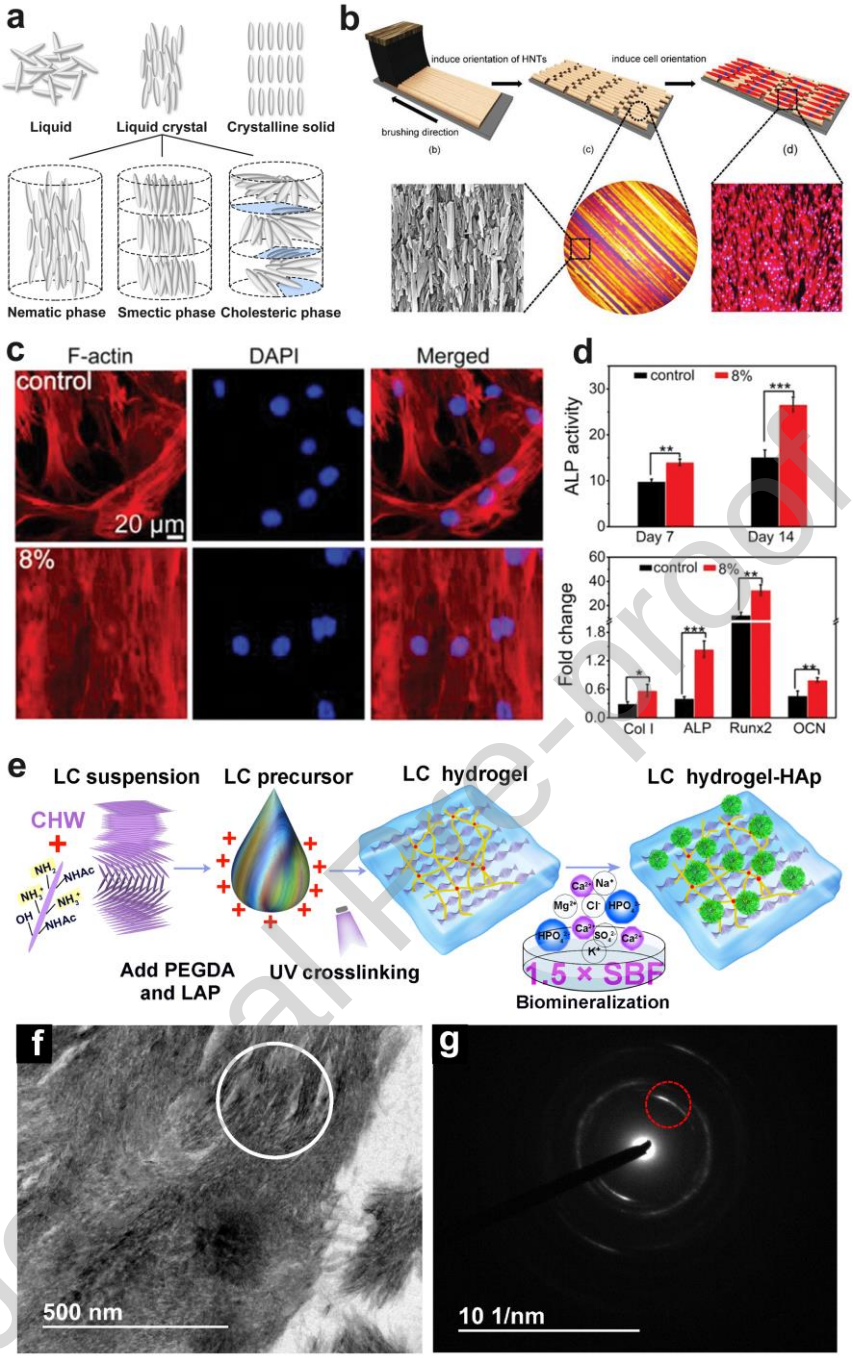

Figure 6. **(a)** Molecular arrangement of liquid, liquid crystal, and crystalline solid, and schematics of three types of liquid crystals. **(b)** Schematic illustration of brush-assisted preparation of ordered halloysite nanotubes for guiding the cell orientation. **(c)** Immunofluorescence staining of hBMSCs cultured on control (glass slide) or 8 wt% aligned halloysite nanotubes (scale bar = 20 μm). Cells showed elongated and aligned morphology and **(d)** higher ALP activity and expression of osteogenic genes on LC halloysite. Adapted from reference[131]. **(e)** Schematic illustration of the preparation and crosslinking interaction of CHW/PEGDA LC hydrogel. Adapted from reference[132]. **(f)** Bright-field TEM images of LC collagen fibrils and the corresponding mineral orientation (dark streaks in the white circle). Scale bar = 500 nm. **(g)** Diffraction patterns generated from the white circles in (f). Scale bar = 10 nm^{-1}. Adapted from reference[69].

Table 1. Summary of advantages and disadvantages of different types of techniques in mimicking bone anisotropic structure.

Technique	Advantages	Disadvantages	References
Natural materials (wood: pinewood, beech wood, etc.)	• Highly aligned and interconnected network like bone; • Rich source and relatively cost-effective; • Naturally, anisotropic mechanical properties.	• Time-consuming and complex delignification; • Lower strength compared to natural cortical bone; • Uncontrollable microstructure.	[89 – 93]
Surface patterning (embossing, moulding, microimprinting, etc.)	• Simple fabrication in 2D and cost-effective; • Good repeatability; • Enhanced scaffold-tissue integration.	• Varied optimal groove spacing across different materials and cell types; • Creating patterns in 3D is complex; • Unknown long-term efficacy; • Grooves may weaken the mechanical properties.	[96 – 99]
Directional freezing	• Produces aligned structures without introducing additional chemicals; • Versatility in crosslinked gels and liquid solution; • Preserves bioactivity of materials during the process.	• Poorer alignment on crosslinked polymers; • Contradictions between directional freezing and crosslinking; • Energy-consuming.	[103 – 112]

Micro self-assembly and Liquid crystals (nano self-assembly)	• May represent the closest to the process bone formation ; • Potentially the strongest biological activities.	• Technical difficulties in appropriate control methods; • Currently challenging to scale up for applications; • Poor particle control may lead to variations in scaffold properties; • Limited types of materials; • Unable to reach comparable strength as bone.	Micro self-assembly [113 – 117]; Liquid crystals [69], [131], [132], and [134]
Electrospun fibres	• Easy alignment and film fabrication; • Convenient and versatile in secondary processing.	• Less feasible to be fabricated into stiff and thick scaffolds; • Insufficient bioactivity of poly(ε-caprolactone) (PCL) substrates; • Unable to be used at load-bearing sites.	[118 – 121]

3.7 Potential mechanisms of anisotropic structure-enhanced osteogenesis

The anisotropic structures facilitate osteogenesis via multiple mechanisms. 1) The first key mechanism may be improved diffusion/infiltration. A research group fabricated nano-HAp/polyamide66 (n-HAp/PA66) scaffolds with axially aligned channels (300 μm) utilising the thermally induced phase separation (TIPS) technique[135]. They found that the MG63 cells were elongated along the direction of channels of the anisotropic scaffolds and infiltrated better into the inner space. The in vivo experiment on rabbit radius bone defect models demonstrates that compared to isotropic scaffolds, anisotropic scaffolds facilitate new bone ingrowth and vessel invasion. The anisotropic structure improves mass transport and eliminates a necrotic core, while scaffolds with random pores generally have lower interconnectivity and a 'bottleneck' effect between adjacent large pores. Earlier computational diffusion models[136-138] indicate that the effective diffusion decreases with increasing tortuosity or disordered microstructures. A more distorted structure hinders the effective exchange of mass; hence, isotropic scaffolds frequently encounter issues of tissue ingrowth. This may be one of the reasons that simply 'porous' scaffolds do not always ensure satisfactory regenerative outcomes, especially in large bone defects, which require a larger volume of scaffolds. 2) Apart from

directly regulating bone cells, anisotropic topography can also guide the behavior of immune cells, which improve the immune microenvironment for enhanced osteogenesis. In a study[139], researchers used fish scales with anisotropic ridged micropatterns to polarise M2-phenotype macrophages, which in turn increased the secretion of the anti-inflammatory cytokines IL-4 and IL-10. The improved immune microenvironment together with MCFs further synergistically accelerated the osteogenic differentiation of rabbit BMSCs via the Wnt/β-catenin pathway. 3) Mediating cell morphology, cytoskeletal tension, and orientation. A study investigated the effects of micro- or nanoanisotropic lines on hMSC behaviours [140]. The authors found that the cells were elongated along the direction of parallel patterns, showed enhanced attachment and osteogenesis, and had upregulated expression of adhesion- and calcium-related genes. Additionally, stem cells exhibited a stronger response in terms of morphology, proliferation and differentiation on nanopatterns than on micropatterns. Reorganising the cytoskeleton and elongating the nuclei are crucial for signal transduction during transdifferentiation. Previous research has linked the elongation of the cytoskeleton and nucleus to modifications in gene expression patterns and cellular differentiation[141-143]. Elongation of both the cytoskeleton and nucleus is a frequently observed phenomenon in cells with enhanced osteogenesis. 4) Multiple molecular pathways may be involved. Liu *et al*.[144] designed a 4D-printed biomimetic periosteum with a hydrogel outer layer and an aligned hMSC sheet inner layer. They identified that the anisotropic micropatterns promoted migration, angiogenesis, and osteogenic differentiation of hBMSCs via activation of the PI3k/Akt signalling pathway. The PI3k/Akt pathway is actively involved in the mediation of cytoskeletal dynamics[145]. Furthermore, the designed anisotropic periosteum induced better new bone integration with the host tissue in the nude mouse calvarial defect models, showing a facilitating effect on angiogenesis and tissue ingrowth. In another paper[146], the authors cultured cells on repetitive nanotopological pillars with a size gradient and observed that nanotopological signals stimulated FAK, a protein that responds to integrin and is responsible for triggering mechanical transduction. This activation further led to downstream protein activation of ERK and JNK, which then resulted in the movement of transcriptional coactivator with PDZ-binding motif (TAZ) from the cytoplasm into the nucleus. TAZ activation of RUNX2 in the nucleus then upregulated osteogenesis. Noticeably, the regulation of ERK by FAK may also occur through the PI3k/Akt signaling pathway. Similarly, Yang *et al*.[147] cultured hBMSCs on hydroxyapatite surfaces treated using sandpaper to create parallel lines and varying roughness. The results demonstrated that hBMSCs displayed oriented morphology and had increased expression of YAP/TAZ and osteogenic-related genes such as RUNX2, OPN, and ALP. These findings suggested a signaling pathway affected by surface topography: the integrin recognises the surface, and the signal transduces to Rho-GTPase, which increases the tension of F-actin and squeezes the nucleus. This results in the movement of YAP/TAZ from the cytoplasm to the nucleus, where they activate osteogenic-related genes. Zhang *et al*.[148] indicated that by comparing MC3T3-E1 cells on grid patterns and flat surfaces, it was found that the cells had a larger spreading area and higher YAP expression on the grid pattern. After silencing YAP, ALP expression was attenuated. Additionally, on rougher surfaces, focal adhesions were distributed on the edge of cells, and the rough surface facilitated the nuclear localisation of TAZ, which then led to enhanced osteogenesis[149]. Based on the current literature, a potential signalling pathway for anisotropic pattern-induced enhancement of osteogenesis was hypothesised. The

hypothesis suggests that at the very beginning, cells recognise surface patterns and manoeuvre the location of integrin and focal adhesion aggregation, which leads to cell spreading. The bound integrin then transduces the topographical signal to FAK, leading to downstream activation of Rho-GTPase and an increase in cytoskeleton tension. The high tension squeezes the nucleus, inducing YAP/TAZ movement from the cytoplasm into the nucleus, where they upregulate RUNX2, a key gene for osteogenesis. It is important to note that this hypothesis is based on the current published literature, and it may not be applicable in all situations. In particular, the complexity of the natural ECM makes it difficult to study these factors at the cellular level. The cell-substrate interaction in bone development and regeneration should be mediated not only by a single factor (anisotropic patterns) but also by a collective result of topography, roughness, matrix stiffness, dynamic stimuli and so forth. Furthermore, multiple signaling pathways are likely involved in this complex process of cell communication, apart from the currently identified PI3k/Akt pathway. Further studies and clarification of how these pathways co-op and crosstalk with others to enhance osteogenesis on anisotropic substrates are needed.

3.8 Conclusion and future perspectives

The anisotropic structure is a unique form found in many organisms, including human cortical bone. The microstructure of bone plays an important role in mechanical transduction and biological guidance. This review presents current studies and understanding of anisotropic structure-induced directional osteogenesis. In bone tissue engineering, researchers have developed various materials and techniques to mimic the anisotropic arrangement, resulting in upregulated osteogenic genes and proteins. However, the ideal technique has not yet been found. Each of them demonstrates significant advantages and disadvantages. Most of their controllability remains at the microscale, lacking nanoscale cues for directing cells. Additionally, there is limited information regarding their capability of inducing directional osteogenesis. Although many studies have reported increased osteogenic gene expression levels and enhanced calcium deposition, there is insufficient evidence to demonstrate that newly formed minerals have achieved long-range ordered structures at multiple scales. The key factors in manipulating calcium secretion patterns are largely unknown. The randomly formed and distributed calcium poses a major obstacle for more controllable bone repair and for growing bone in laboratories. We also summarised findings regarding the signalling pathway in anisotropic structure-enhanced osteogenesis, but the available information is still limited. More studies are needed to further fill in the knowledge gap of cell-substrate interactions to guide lab-based scaffold and artificial bone fabrication.

The understanding of anisotropic structures and their impact on bone tissue engineering has laid the foundation for promising future research and advancements. While various materials and techniques have been developed to mimic the anisotropic arrangement found in cortical bone, there is still a need to discover an ideal technique that can precisely direct cells towards the osteogenic lineage. To achieve this, researchers should further investigate the intricate interactions between anisotropic structures and bone cells. A deeper understanding of how these structures influence cell proliferation and differentiation towards osteogenesis will enable the development of more effective strategies. Anisotropic structures, especially LC structures, hold

potential as templates for guiding the growth direction of bone tissue in vitro. This approach could potentially lead to great development of advanced bone grafts and implants that promote directional osteogenesis and enhance regenerative outcomes. In parallel, the development of new materials and techniques that better mimic the anisotropic microstructure of bone should be explored. Researchers can investigate the integration of 3D printing, photoresponsive materials, magnetic or electric fields, and other innovative approaches to achieve precise control over anisotropic structures. These advancements would facilitate the fabrication of complex and reproducible structures, enabling more effective bone tissue engineering. Furthermore, the manipulation of calcium secretion patterns, a critical factor in achieving controllable bone repair, needs to be thoroughly investigated. Understanding the underlying mechanisms of calcium formation, distribution, and organisation within anisotropic structures is crucial. By gaining insights into these processes, researchers can devise strategies to overcome the challenges posed by randomly formed and distributed calcium. This knowledge will facilitate the development of more precise bone regeneration techniques in laboratory settings. Moreover, the signaling pathways involved in anisotropic structure-induced osteogenesis require further exploration. More comprehensive studies are needed to elucidate the intricacies of these pathways and their interactions with anisotropic structures. This understanding will be instrumental in developing targeted approaches that harness signalling pathways to enhance bone regeneration and repair.

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Author Contributions: Conceptualisation, data collection, writing—original draft preparation, writing—revision, J. Chen. All author has read and agreed to the published version of the manuscript.

Funding: The author would like to thank the support by the UCL Global Engagement Fund (579149), UCL Student Activities Participation Fund, UCL Robert Brown Award, and the Armourers & Brasiers' Gauntlet Trust Travel Grant.

Declaration of interests: The author declares that there has no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements: The author thanks Prof. Wenhui Song at University College London for beneficial comments on the manuscript.

CRediT author statement

Conceptualisation, data collection, writing—original draft preparation, writing—revision, J. Chen. All author has read and agreed to the published version of the manuscript.

Declaration of interests

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☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights:

- Techniques addressing rising bone defect challenges.
- Unveiling cortical bone's unique anisotropic structure.
- Exploring anisotropic designs for enhanced osteogenesis.
- Advocating deeper research for anisotropy-enhanced bone regeneration.