

Bone organoid 3D bioprinting: construction and application in bone regeneration

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Abstract: Repair of critical-size bone defects remains a significant challenge. In recent years, organoid technology has emerged as a promising strategy for bone regeneration, but developing bone organoids with complex structure and function still faces many hurdles. Three-dimensional (3D) bioprinting, a revolutionary additive manufacturing technology, can precisely deposit bioink layer by layer with a specific pattern to construct the required 3D structure, which provides a simpler and more efficient method for generating bone organoids. This review summarizes the 3D bioprinting strategies of bone organoids and their application in bone regeneration in order to provide a reference for future research.

1 Introduction

The treatment of critical-size bone defects caused by tumors, trauma or infection remains challenging. Autologous bone graft is considered the gold standard therapy, but it has the disadvantages of limited supply and high incidence of complications. As an alternative to autografts, allografts carry the risk of disease transmission and immunological rejection. Although various bone substitutes have been developed, they still need to be improved in mechanical properties, biodegradability and osseointegration [1]. In recent years, organoid and three-dimensional (3D) bioprinting technologies have shown great potential in regenerative medicine. Bone organoids are self-organized 3D micro-bone tissues derived from stem cells or progenitor cells, which provide a new approach for bone regeneration [2]. Compared with traditional organoid construction strategies, 3D bioprinting allows for the generation of more physiologically relevant bone organoids through precise assembly of cells, biomaterials, and growth factors (Figure 1) [3]. This review focuses on 3D printing strategies for bone organoids and their applications in bone regeneration. Furthermore, the current challenges and future development of bone organoid bioprinting are discussed.

2 3D bioprinting of bone organoids

2.1 3D bioprinting techniques

3D bioprinting is an additive manufacturing technology that enables precise layer-by-layer deposition of bioink based on a computer-aided design file to create 3D constructs [4]. Commonly used 3D bioprinting techniques include inkjet bioprinting, extrusion bioprinting, laser-

assisted bioprinting, and light-curing bioprinting, which are compared in Table 1. The schematic of the four bioprinting techniques are shown in Figure 2.

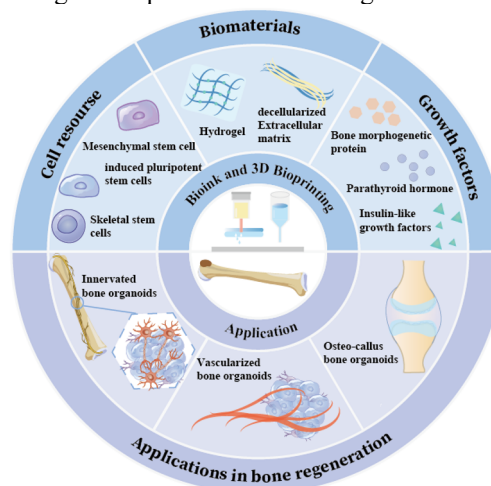


Figure 1. Bone organoid 3D bioprinting for bone regeneration

Inkjet bioprinting originates from commercial 2D inkjet printing, which sprays bioink onto the substrate in the form of droplets to construct the desired shape. Droplets are usually generated by thermal or piezoelectric actuation [5]. The advantages of inkjet bioprinting include fast print speed, high resolution and high cell viability. In addition, inkjet bioprinting can integrate various biomaterials and cells through multiple nozzles to create complex organoids. However, this technique is unable to print high viscosity bioinks due to its low driving force [6]. Extrusion bioprinting overcomes this problem by extruding bioink mechanically or pneumatically to form continuous filaments. It provides wider material compatibility and allows for the creation of organoids with high cell densities. However, the high shear stresses during the printing process can also cause

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damage to cells [7], so shear-thinning bioinks are more favored.

Unlike the above two approaches, laser-assisted bioprinting doesn't use nozzles to eject bio-ink, thus avoiding the problems of clogged nozzles or shear stress affecting cell viability. This technique utilizes laser pulses to focus on the energy absorbing layer, which generates a high-pressure bubble and then propels bioink droplets towards the receiving substrate. It is compatible with a wide range of bioinks and its high resolution allows printing of individual cells. Although this technology has many advantages, the effects of laser on cells have not been fully investigated. Besides, this bioprinting system is costly and the printing process is time-consuming [8]

Light-curing bioprinting uses ultraviolet or visible light to selectively solidify photosensitive polymer materials layer by layer, which can be categorized into stereolithography (SLA) and digital light processing (DLP) depending on the way of light scanning. SLA printing uses a laser beam to cure the photosensitive resin in a point-by-point manner and then produce a specific shape. DLP provides faster print speed as it cures an entire layer at a time using a liquid crystal display or a digital micromirror device. But both of them are still based on light-curing technology, with limited material options and applications. In addition, the curing process usually requires the use of photoinitiators, which may produce potential cytotoxicity [9].

Table 1. Comparison of major 3D bioprinting techniques

Bioprinting technique	Advantages	Disadvantages
inkjet bioprinting	<ul style="list-style-type: none"> ●high resolution ●fast print speed ●low cost ●multi-material printing 	<ul style="list-style-type: none"> ●nozzle clogging
extrusion bioprinting	<ul style="list-style-type: none"> ●fast print speed ●wide material compatibility ●multi-material printing 	<ul style="list-style-type: none"> ●limited resolution ●low cell viability
laser-assisted bioprinting	<ul style="list-style-type: none"> ●high resolution ●without nozzle clogging 	<ul style="list-style-type: none"> ●slow print speed ●high cost
light-curing bioprinting	<ul style="list-style-type: none"> ●high resolution ●fast print speed ●without nozzle clogging 	<ul style="list-style-type: none"> ●potential cytotoxicity ●bioink waste

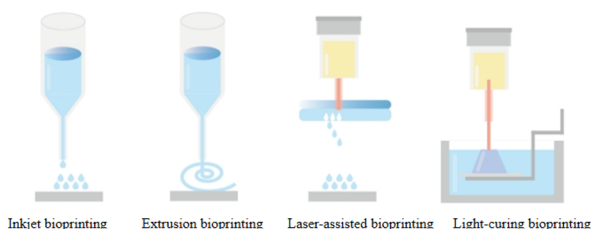


Figure 2. Four major 3D bioprinting technologies

2.2 Bone organoid bioinks

Bioink is an important part of 3D bioprinting, which typically consist of cells, biomaterial scaffolds, growth factor, etc.

2.2.1 Biomaterials

The biomaterials used for 3D bioprinting should have good biocompatibility, bioactivity so as to provide a favorable microenvironment for the growth and differentiation of various cells. The bioink material should also have mechanical strength that could mimic bone tissue and good printability. Hydrogels are commonly used materials for bioprinting, including natural polymers such as alginate, gelatin, collagen, hyaluronic acid, and synthetic polymers such as polyethylene glycol, polyvinyl alcohol, polylactic acid [10-12]. However, these materials are still unable to replicate the complex bone microenvironment. Decellularized extracellular matrix (dECM) has recently attracted a lot of attention. dECM is obtained by physical, chemical, or enzymatic decellularization of natural tissues, which retains tissue-specific structure and biochemical cues [13]. Due to the poor mechanical properties and shape fidelity, the application of dECM requires further modification or combination with other polymers [14].

Considering the inorganic/organic components of bone matrix, inorganic fillers are often incorporated into bioinks to enhance mechanical properties and osteoinductive activity. Bioceramics such as hydroxyapatite (HAP) and beta-tricalcium phosphate (β -TCP) are widely used due to their similar chemical compositions to the inorganic phase of bone [15]. In addition, other nanoparticles such as silicate nanoparticles and graphene oxide have been explored as functional inorganic fillers. A study has developed a graphene oxide/alginate/gelatin bioink, and bone organoids produced with this bioink exhibited significant mineralization and lacuna-canalliculi network formation with long-term mechanical loading [16].

2.2.2 Cell sources

Various stem cells and progenitor cells have been used for developing bone organoids. Mesenchymal stem cells (MSCs) are the most commonly used stem cells, which can be isolated from many tissues such as bone marrow, adipose tissue and dental pulp [17]. Skeletal stem cells (SSCs) are another type of stem cells existing in bone marrow, periosteum, and growth plates. SSCs possess higher bone regenerative potential and can be efficiently induced into bone organoids [18]. However, adult stem cells still have some limitations, including limited sources and susceptibility to senescence [19]. An alternative approach for constructing bone organoids involves induced pluripotent stem cells (iPSCs). iPSCs can be generated from patients' somatic cells and exhibit multidirectional differentiation and long-term self-

renewal ability, which makes them a promising cell source for bone organoids^[20].

2.2.3 Growth factors

Osteogenic differentiation is regulated by multiple signal pathways, such as Wnt/ β -Catenin signaling pathway, notch signaling pathway, and BMPs/TGF- β Pathway. Critical signaling factors can be added to bioinks to promote osteogenic differentiation, among which BMPs are the most representative^[21]. Moreover, controlling the spatial and temporal distribution of bioactive factors can lead to multicellular differentiation, thus producing more complex organoids like osteochondral organoids. For example, Kilian *et al.* added nanoclays into alginate/methylcellulose through core-shell bioprinting. Nanoclays were loaded with BMP-2 and TGF- β 3 respectively as the “core” and accurately deliver them to the cells of corresponding “shell” to drive osteogenic/chondrogenic differentiation, which provides ideas for the development of multicellular bone organoids^[22].

3 3D bioprinted bone organoids for bone regeneration

Bone organoids have the potential to revolutionize the therapeutic strategy for bone defects. In 2024, Wang *et al.* designed a MSCs-laden gelatin methacrylate/alginate methacrylate/hydroxyapatite (GelMA/AlgMA/HAP) bioink, which can be printed via DLP for mass production of self-mineralizing bone organoids. Multicellular differentiation and trabecular bone-like structures was observed in bone organoids after *in vivo* implantation, which formed a highly complex bone microenvironment. They reported that the bone organoids significantly promoted bone regeneration within 8 weeks^[23]. Endochondral ossification is an important mechanism of bone development and regeneration, therefore, constructing cartilaginous callus organoids based on developmental engineering concepts is another strategy for bone regeneration. In 2022, Xie *et al.* obtained MSCs-loaded gelatin methacrylate hydrogel microspheres by DLP printing. These cell microspheres exhibited endochondral ossification gene expression patterns during chondrogenic induction and gradually transformed into osteo-callus organoids, which could achieve rapid bone regeneration within 1 month^[24]. In another study, Bolander *et al.* induced human periosteum-derived cells into three stages of early chondrogenesis, late chondrogenesis, and osteogenic differentiation through the control of growth factors and oxygen conditions, then they developed three kinds of thiol-ene alginate hydrogels with different stiffness to mimic the ECM of the corresponding differentiation regions in callus. Finally, modular callus organoids were assembled by extrusion 3D printing, which provides ideas for the further bionic development of bone organoids^[25].

In order to ensure the survival of bone organoids after implantation, it is also necessary to reconstruct

vascular networks to facilitate oxygen and nutrient delivery as well as waste removal. Sacrificial materials are widely used in printing perfusable vessel channels. For example, Amler *et al.* used hyaluronic acid methacrylate bioink with human umbilical vein endothelial cells (HUVECs) to print channel structures in bone organoids, then hyaluronic acid was digested and the released cells attached to the channel walls to form vascular networks^[26]. Coaxial bioprinting is another method to produce vascularized tissues. In 2023, Zhang *et al.* used GelMA/Alg bioink loaded with MSCs and HUVECs as the outer-layer and middle-layer bioink respectively, and a calcium chloride (CaCl₂) solution was used as the inner-layer bioink. The CaCl₂ solution crosslinked with the middle layer bioink during the printing process and formed hollow nutrient channels, then a stable large-scale vascularized construct was obtained^[27].

Nerve fibers also play important roles in bone development and regeneration, and introducing innervation into bone organoids can facilitate the structural and functional recovery of bone defects. In 2022, Zhang *et al.* constructed an innervated bone organoid by dual-channel printing of Schwann cells and MSCs. They added calcium silicate (CS) nanowires into GelMA bioinks, and bioactive Ca and Si ions released by CS significantly promoted new bone formation and ingrowth of nerve fibers *in vivo*^[28].

4 Conclusions and future perspectives

Great progress has been made in bone organoid bioprinting recently. Through precise manipulation of cells and microenvironmental cues, 3D constructs that closely mimic native bone tissues can be produced. However, organoid bioprinting is still in early stages and faces many challenges. Firstly, current 3D printing technologies have various limitations, and the printed bone organoids cannot fully reproduce the real structure and complex vascular networks of bone tissues. Some more advanced 3D bioprinting techniques have been developed, for example, embedded bioprinting has been used to construct hierarchical vascular networks with varying diameters, showing great application prospects in producing vascularized organoids^[29]. In terms of bioinks, the mechanical properties and fidelity of hydrogel bioinks still need to be improved. Additionally, the decellularization protocols and evaluation criteria of dECM bioinks should be standardized before clinical application^[30]. More and more studies have also focused on the development of nanocomposite bioinks, which exhibit excellent properties such as mechanical stability, shear-thinning properties, promoting mineralization, and precise delivery of bioactive factors^[31]. Moreover, it's noteworthy that AI shows great potential in optimizing bioink formulations and predicting printability, so combining AI with 3D bioprinting is one of future trends^[32].

With the development of smart biomaterials, the concept of four dimensional (4D) bioprinting has emerged, which adds a temporal dimension to 3D

bioprinting and enables the printed 3D structures to change their shape and function in response to stimuli [33]. In the future, 4D printed bone organoids are expected to fit in irregular bone defects easily through shape transformation and improve cell maturation through dynamic tissue restructuring [34, 35]. In addition, 4D printed self-folded microtubes can be used to create vascular structures in bone organoids [36]. By combining suitable bioinks with advanced bioprinting techniques, more physiologically relevant bone organoids can be produced to meet the clinical needs for effective bone repair.

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