Advances in Three-Dimensional Bioprinting for Hard Tissue Engineering

Sang-Hyug Park¹, Chi Sung Jung^{2,3}, Byoung-Hyun Min^{2,3,4*}

¹Department of Biomedical Engineering, Pukyong National University, Busan, Korea

²Department of Molecular Science & Technology, Ajou University, Suwon, Korea

³Cell Therapy Center, Ajou University Medical Center, Suwon, Korea

⁴Department of Orthopedic Surgery, School of Medicine, Ajou University, Suwon, Korea

The need for organ and tissue regeneration in patients continues to increase because of a scarcity of donors, as well as biocompatibility issues in transplant immune rejection. To address this, scientists have investigated artificial tissues as an alternative to transplantation. Three-dimensional (3D) bioprinting technology is an additive manufacturing method that can be used for the fabrication of 3D functional tissues or organs. This technology promises to replicate the complex architecture of structures in natural tissue. To date, 3D bioprinting strategies have confirmed their potential practice in regenerative medicine to fabricate the transplantable hard tissues, including cartilage and bone. However, 3D bioprinting approaches still have unsolved challenges to realize 3D hard tissues. In this manuscript, the current technical development, challenges, and future prospects of 3D bioprinting for engineering hard tissues are reviewed.

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Key Words: Three-dimensional bioprinting; Hard tissue engineering; Cartilage; Bone; Tissue regeneration

INTRODUCTION

The development of implantable tissue and organ transplantation has rapidly changed the scope of medical treatment to satisfy unmet clinical needs [1]. Tissue engineering requires a multidisciplinary approach to meet the demand to repair critically damaged tissues that have a poor capability to regenerate spontaneously, such as bone and cartilage [2]. These tissue engineering strategies commonly require the combination of cells and biomaterial scaffolds for implantable engineered tissues and organs that can regenerate physiological functions. Despite some initial successes in engineering relatively simple tissues, many challenges remain in developing tissues and organs suitable for clinical transplantation [3-5]. An important component in tissue engineering is the functional scaffold. A variety of fabrication techniques have been developed that successfully regenerate complex and functional tissues. Popular methods used include gas foaming, phase separation, salt leaching, and freeze drying

[6]. Conventional tissue engineering has demonstrated success in generating bone and cartilage, but achieving reproducible results has proved challenging. The one of limitations of these conventional scaffold-based approaches include the intrinsic inability to mimic the complex architectures that are required for clinical use [1,3]. The recently developed three-dimensional (3D) printing technology promises to bridge the difference between artificially engineered tissues and native tissues. This 3D printing is rapidly emerging as a key scaffold fabrication strategy for mimicking native tissue complexity. In practice, layer-by-layer stratification of 3D printing can more precisely deliver different cells or mechanical cues in the designed 3D architecture than cannot be achieved by conventional fabrication methods [7,8]. Researchers have demonstrated the possibility of recreating engineered constructs with accurate, detailed, and even personalized features that mimic native architecture [9-12]. When applied to hard tissues, 3D printing provided a favorable microenvironment for bone formation. In addition, 3D printing was used to generate complex shaped cartilage tissue that possessed the properties of human cartilage [11]. Despite the strong potential of 3D printing for tissue regeneration, many challenges still remain, including printing resolution, speed, and processes for bioprinting. In addition, the simple 3D printing technique cannot reproduce the various scales and complexities

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***Corresponding author:** Byoung-Hyun Min, Department of Orthopedic Surgery, School of Medicine, Ajou University, 164 World cup-ro, Yeongtong-gu, Suwon 16499, Korea.

Tel: 82-31-219-5225, Fax: 82-31-219-4442, E-mail: dr.bhmin@gmail.com

of engineered tissues. Thus, this review will discuss the current technical development of 3D printing in hard tissue fabrication.

REGENERATIVE MEDICINE AND HARD TISSUE ENGINEERING

The study of regenerative medicine has contributed new treatments for patients with aging diseases of the musculoskeletal system [13,14]. Regenerative medicine requires the combination of engineering and biology for the generation of functional tissue constructs [15]. Non-repairable hard tissue defects can be congenital or acquired from trauma, infection, inflammation or tumors. The reconstruction of these defects is challenging due to the unpredictable outcomes. For several years, hard tissue engineering focused on alternative treatment options for tissue defects. The main strategy was to generate 3D constructs that mimic natural tissue in order to achieve functional and cosmetic tissue formation once implanted. Currently, various engineered grafts for tissue replacement are available. The application of engineered grafts has a high clinical potential for filling these defects, since the bone and cartilage grafts do not have to be harvested from donors. Although engi-

neered grafts are produced in simple geometries, various materials have been used as scaffolds, such as polymers, ceramics, and hydrogels [13,14].

WHY WE NEED A 3D PRINTING TECHNOLOGY FOR HARD TISSUE FABRICATION

Scaffolds fabrication is a major part in tissue engineering. An ideal scaffold should be biocompatible, biodegradable, and have adequate physical and mechanical properties. Some natural and synthetic biocompatible bone substitutes have been developed to promote the bone regeneration, as alternatives to autologous or allogeneous bone grafts [16]. Although there has been much development in hard tissue regeneration, one of the primary challenges is optimizing patient-specific treatment. Additive manufacturing (AM), particularly 3D printing, is emerging as a powerful tool for reproducing the mechanical, topological, and cellular properties of tissue [17,18]. To create a suitable 3D scaffold, 3D printing is thus overcoming the drawbacks of conventional tissue engineering, and emerging as an advanced alternative to autologous grafting and organ replace-

Figure 1. Entire process for 3D bioprinting using patient medical images. Implantable printed tissues are fabricated by layered stratification of material, hence rendering a 3D volumetric structure. The structures are designed using computer-aided design software, or from patient medical images. The selection of biomaterials and cells is essential for the 3D tissue printing. 3D bioprinting systems, including inkjet, laser-based, or extrusion printers have to utilize for the printing of biomaterial bioink with/without selected cells to form a hard tissue structure. 3D: three-dimensional.

ment [19]. In addition, 3D printing has shown promising results for use in personalized regenerative medicine. The combined use of anatomical 3D image analysis and computed tomography (CT) techniques can achieve tailored treatment for tissue defects [20]. Organ/tissue printing now enables accurate 3D organization of components such as cells, biomaterials, and biomolecules in a way to mimic the structure of native tissues [21]. Scaffolds generated by 3D printing layer by layer can have complex geometries such as undercuts, curvatures, and interconnecting channels, while maintaining a relatively high-throughput fabrication process [22].

The ultimate goal of 3D printing technology based on layer-

by-layer stratification is to create hard tissue substitutes that have qualities similar to human tissues, and can be implanted to quickly repair bone and cartilage loss. Although the development of printed grafts that match the shape, size and type of defect is challenging, 3D printing has significant potential for advanced regenerative therapy [23]. It is important to understand the biological environment to fabricate tissues and organs using 3D printing. Since cells can interact with the substrate matrix in printed 3D spaces. However, a major hurdle for tissue or organ printing is the gradual and successful transformation of the post printing structure to functional tissue constructs. The cell response, stability of the structure, and ECM deposition are

Table 1. Summary of commercialized 3D printed products for applications of hard tissue repair

3D printing	Product	Company	Printing material	Target tissue
Electron beam melt	Tesera trabecular technology	Renovis	Titanium alloy	Bone plate
	Bone tether plates	MedShape, Inc.	Titanium	Bone plate
	EXACTECH	InteGrip®	Plasma-sprayed titanium	Cortical bone
	EndoLIF® On-Cage implant	Joimax	Titanium alloy	Spine
	Lumbar PLIF cage	Advanced medical technologies	Titanium	Spine
	Hip joint implant	Ak medical	Titanium	Knee
	Hip cups	National Rehabilitation Aid Research Centre	Titanium	Hip
Selective laser melting	Adler ortho, lima	Acetabular cup	Titanium	Knee
	Jaw bone implant	3D Medical Limited	Titanium	Jaw bone
	Spinal cages	EndoLIF	PEEK or titanium alloy	Spine
	SpineFab VBR System	Oxford Performance Materials	OXPEKK® polymer	Spine
	Denture	DENTCA	Methacrylate-based resin	Dental
	Knee trays	-	Ti 6Al 4V alloy, Co-Cr-Mo alloy	Knee
	Bego	Wirobond® C	Base metal alloy	Hip
Selective laser sintering process	OsteoFab® Patient Specific Facial Device	Oxford Performance Materials	OXPEKK® polymer	Craniofacial bone
	Skull patch		Titanium	Craniofacial bone
	Screws	Medshape	Ti 6Al 4V alloy	Surgery instrument
Additive manufacturing	Osteomesh/osteoplug	Osteopore	Polycaprolactone	Craniofacial bone
	CMF mesh	T&R biofabrication	Polycaprolactone	Craniofacial bone
	FastForward™ astForwardl bonete	MedShape, Inc.	Medical grade titanium alloy (Ti-6Al-4V)	Bone plate
	SurgiCase Orthopaedics	Materialise N.V.	-	Surgery instrument
Digital light processing	E-Dent	Envision TEC	Acrylic resin	Dental
	Hearing aids		Custom patient solution	Ear
Direct metal laser sintering	LayerWise	Titanium orthopedic, maxillofacial, spinal, and dental implants	Medical-grade titanium	Deantal
Fused deposition modeling	ReDura	Medprin	Poly(L-lactic acid)	Duramater

3D: three-dimensional, PLIF: posterior lumbar interbody fusion, VBR: vertebral body replacement, CMF: craniomaxillofacial fracture

important in tissue printing [24]. In the last ten years, researchers have demonstrated the potential of 3D printing technologies for the fabrication of functional tissue constructs [4,14,25,26]. Therefore, we believe that 3D printed hard tissues will overcome many of the shortcomings of bone and cartilage grafts and transitional tissue engineering.

CURRENT 3D PRINTING TECHNIQUES FOR HARD TISSUE FABRICATION

3D printing is currently the most advanced method of AM for tissue engineering. In order to be effective, 3D bioprinting must realize the capacity of precise positioning, sourcing of suitable printable biomaterials, and delivering cell sources to the desired targeted positions. In addition, maturation after printing is also crucial to engineering functional tissues [27]. As shown in Figure 1, objects are fabricated by layered stratification of material, hence rendering a 3D volumetric structure. The printed structures are designed using computer-aided design (CAD) software, or from images obtained via CT, or magnetic resonance or X-ray imaging. Traditionally, 3D printing trials have been primarily utilized to fabricate scaffolds constituted of synthetic inks, which are then seeded with living cells and tested *in vivo* after implantation. More recently, direct printing of living cells, cell-laden biomaterials, and scaffold-free cell aggregates has been increasingly studied at a much greater level of complexity [28,29]. Although many types of printers are available commercially, currently, most systems are inkjet, laser-based, or extrusion printers. Common to all of these systems is coordinated motion of stages in the X, Y, and Z directions, while an automated system dispenses a bioink via different mechanisms [18]. These innovative AM technologies are able to print the 3D structure directly from CAD data to match the individual specific tissue. Current research into AM technologies includes stereolithography (SLA), selective laser sintering (SLS), fused deposition modeling (FDM), and extrusion-based plotting (EBP). In particular, an FDM based printer was used to fabricate a 3D lumbar cage for lumbar interbody fusion-based on computational and experimental analysis. This study demonstrated the advantage of the 3D printing technique for customized implants for various medical applications [30]. To date, commercially available 3D-printed implants have been fabricated using conventional 3D printing methods such as SLA, SLS, and FDM. T&R Biofabrication (Siheung, Korea), markets a poly(ϵ -cap-rolactone) (PCL) 3D printed product for application in craniofacial reconstruction (Table 1). For hard tissue engineering, currently, most applications are for bone and cartilage 3D printing using EBP, ink jet printing, and SLA [31-33].

EXTRUSION-BASED PLOTTING

EBP is a rapid manufacturing technique. In order to print viscous materials, extrusion plotting uses either an air force pump or a mechanical screw plunger to dispense bioinks. Therefore, this method is referred to as direct printing. The merit of extrusion-based 3D printers is its accuracy in depositing materials and cells to complex structures [34]. An EBP applicable material requires rheological properties that allow its extrusion through a small needle and fast stabilization after deposition to guarantee shape fidelity of the extruded line [35]. The 3D plotter is capable of using and changing combined bioink to realize the designed scaffold structure. The dispensers utilize a pneumatic or screw pressurized system to extrude the material from the bioink containers. Types of dispensers in current extrusion systems can vary [8]. Pneumatic micro nozzles powered by compressed gases support a wider range of viscosity, however they have difficulty precisely controlling the deposited mass. In addition, the shear stress from the various-sized nozzles can impact negatively on cell viability during the printing process. Screw-based nozzles can print without inlet air and are less expensive, but they experience problems dispensing high viscosity materials [36]. Fedorovich et al. [19] used this technique to print bone marrow stem cells on to photo-polymerized lutrol, agarose, and alginate. Hydrogels that are solidified through either thermal processes or post-print cross-linking are being used for printing cells to produce various hard tissues (cartilage and bone) using various materials such as collagen, chitosan, alginate, agarose, gelatin and decellularized ECM (dECM) [37]. Despite many benefits, EBP has certain disadvantages compared to other 3D printing technologies. The precision of the printing resolution is considerably restricted [38]. In addition, the bioink undergoes physical stress during the extrusion process [36]. Thus, candidate hydrogels are limited. The use of harsh cross linking agents is also of concern, as printing process must be cytocompatible. Recent studies have used a narrow range of materials such as alginate gel and lutrol F127 which did not induce the cell differentiation [39].

SLA PRINTING

SLA is based on the layering by laser apposition of photosensitive hydrogel polymer and cells. After each layer the construct is solidified by exposure to light or laser. SLA printing allows fabrication of internal pores, defined macroscopic features, and strong constructs required for scaffolds used for repair of complex tissue geometries. A hybrid hydrogel of chitosan and poly(ethylene glycol) diacrylate (PEGDA) was utilized by Morris et al. [40]. Grogan et al. [41] reported that micropatterned gelatin

me-thacrylate (GelMA) scaffolds fabricated using projection SLA printing achieved organized cellular alignment, and promoted meniscus-like tissue formation. However, this type of SLA has the disadvantage of layered stratification that may disable cell contact between layers [42].

INKJET PRINTING

Inkjet printing is one of the most accurate printing technologies to be applied in the field of tissue engineering and regenerative medicine. Similar to 2D printing, it is based on spraying of bioinks using modified printer heads. Targeted cells and scaffolds can be accurately and precisely deposited to desired locations. Therefore, this technology is an ideal approach to fabricate tissues that mimic native anatomic structures. In hard tissue printing, Qu et al. [43] demonstrated the feasibility of thermal inject-based bio-printing technology in the engineering of cartilage tissues. Optimization of bioink parameters is required in order to construct 3D structures to treat a wide variety of cartilaginous lesions. Bioprinting based on thermal inkjet printing technology has proved to have potential in anatomic cartilage engineering. Gao et al. [44] demonstrated the capacity for hard tissue engineering with biomimetic structures using a thermal inkjet printing fabrication. In their study, stem cells suspended in PEGDA and hydroxyapatite (HA) substrates were accurately printed in targeted locations. However, this method does not permit large volumes and strong mechanical property of the 3D printed structure. To overcome these drawbacks, Xu et al. [25] printed hydrogel constructs using ink jet printing with electrospinning in order to improve mechanical property. They demonstrated the feasibility of fabricating zonal cartilage constructs using a combination system. The scaffold generated by combining printing methods demonstrated enhanced mechanical properties compared to conventional hydrogel constructs generated using inkjet printing alone. Printed cells also maintained their viability and produced cartilage specific ECM both *in vitro* and *in vivo*. Cui's group also successfully printed mechanically strong and tissue differentiated bone and cartilage constructs using poly(ethylene glycol) (PEG)-GelMA hydrogel and developed a unique inkjet bioprinting method. This suggests that inkjet printing has promising potential for use in bone and cartilage tissue engineering [45].

DEVELOPMENT OF 3D PRINTING BIOINKS FOR HARD TISSUE FABRICATION

ECM microenvironments are essential to direct the differentiation of stem cells [46]. A main purpose of the 3D printed

scaffolds is the imitation of biological and physical properties for the mimic of native ECM microenvironments [47]. As new printing bioinks are developed and improved printing methods are discovered, the use of 3D printed scaffolds in tissue engineering continues to get more favorable [28]. In recent advances in scaffold-free bioink, tissue derived dECM has been considered as a new source for the 3D tissue printing. Pati et al. [48] utilized dECM components to print tissue analogues, and achieved high cell survival rate and functionality from dECM printed 3D structures. In their recent study, they utilized the fragments of decellularized tissues to print the PCL 3D frame to provide the tissue analogues [49]. Cell laden silk-gelatin bioink has been also developed by same research group for application in extrusion printing systems. Cell compatibility improved in a cell encapsulation *in vitro* culture study [50]. Costantini et al. [51] suggested the high robustness and accuracy of the employed deposition method using ECM biomimetic hydrogel formulated with alginate and ECM analogues for advanced cartilage tissue engineering. Loo and Hauser [52] have also proposed the ideal candidate bioink based on self-assembled peptide which have advantages such as biomimicry, stimuli-responsive properties, biocompatibility, biodegradability, ease of synthesis and functionalisation. They achieved the 3D printing of the dynamic complexity of biological tissue using self-assembling peptide inks. The possibility of 3D printing manufacturing of complex tissue constructs using cartilage tissue derived ECM bioink has been demonstrated [53]. The plotting method was successfully developed for high viscous ECM-based bioink and 3D plotted scaffolds showed highly interconnected pores as well as complex shape. However, ECM bioprinting has not yet been well-established. The effective protocols of tissue decellularization and the cost down strategy of the bioink are needed for the overcoming of limitations. Furthermore, the mechanical properties of dECM are very weak, so there is a need for a frame printed using a hard material to keep the dECM structure from collapsing. Therefore, the printing of scaffolds for reconstruction of tissues that are subjected to higher mechanical loads, such for as bones and cartilage, usually requires the use of ceramic materials or composite scaffolds to improve the scaffold mechanical property [54].

FABRICATION OF BONE AND CARTILAGE TISSUE

Many researches from tissue engineering have been done on bone or cartilage repair. It is because the tissues of bone or cartilage allow us to easily stereotype the supporter with biological materials and to relatively easily control the formation of pores in which we seed or culture the cells. Bone can be divided into

three types according to its morphological and functional characters. That is, it can be divided into the flat bone, the short bone, and the long bone. The flat bone mainly forms the bone of face and skull and formatively plays a role in protecting the important structure of the head. As for the short bone, there are carpal bone of wrist, metacarpal bone, and tarsal bone of ankle

and metatarsal bone...etc., and mainly forming the joint, it enables the movements toward many different directions. Femur, tibia, or humerus...etc., are the examples of the long bone, and they support the weight forming the whole body. According to its histological structure and composition, cartilage can be divided into the hyaline cartilage, the elastic cartilage, and the fi-

Table 2. 3D bioprinting application for cartilage tissue engineering

Cartilage	Printing method	Printing material	Ref. (year)
Cartilage	Extrusion plotting	PCL and decellularized ECM	Pati et al. (2014) [48]
Cartilage	Extrusion plotting	PLA and acrylonitrile-butadiene-styrene terpolymer	Rosenzweig et al. (2015) [88]
Meniscus	SLA	Methacrylated gelatin	Grogan et al. (2013) [41]
Cartilage	Inkjet printing	PEG	Cui et al. (2012) [85]
Ear	Extrusion plotting	PEG/PCL	Lee et al. (2014) [91]
Ear and meniscus	Extrusion plotting	Nanofibrillated cellulose/alginate	Markstedt et al. (2015) [86]
Ear	Integrated tissue-organ printer	PCL polymer and pluronic F-127 with composite gel	Kang et al. (2016) [11]
Knee cartilage	Extrusion plotting	Tissue derived ECM/collagen	Song et al. (2015) [53]
Cartilage	Inkjet printing	Photocrosslinked PEG-GelMA	Gao et al. (2015) [45]

3D: three-dimensional, SLA: stereolithography, PCL: poly(ϵ -caprolactone), PLA: poly(lactic acid), ECM: extracellular matrix, PEG: poly(ethylene glycol), GelMA: gelatin methacrylate

brous cartilage. The hyaline cartilage can be found in the articular cartilage, and it plays a role in minimizing the friction from the joint movements and in distributing the weight. The elastic cartilage has high level of elasticity and there is ear auricle or the cartilage of nasal septum for example. The fibrous cartilage can be found in the incompletely repaired cartilage or the tendinous insertion area. It is sturdy and plays a role in withstanding high level of tensile force. Like this, there are many different types of bone and cartilage according to their morphological and functional characters and each of them plays a very different role. Therefore, in the studies which aim at the repair of bone and cartilage, the selection of biological materials and the manufacture methods should also be very different.

CHALLENGE OF BONE TISSUE PRINTING

Bone is a complex composite of minerals and organic matrix with exquisite structural organization [27]. Although bone is a self-healing tissue [55], there is a critical limit to the amount that can be regenerated naturally [56]. Bone tissue engineering has developed as a promising approach to bone repair and reconstruction. Recently, 3D printing techniques have been applied to bone tissue engineering (Table 2). Yao et al. [57] used anatomic data from CT scans of rabbits to print and test PCL/HA scaffolds that supported physiologically relevant loads. Pati et al. [49] reported the osteogenesis enhanced PCL/poly (lactic-co-glycolic acid) (PLGA)/ β -tricalcium phosphate (β -TCP) printed scaffolds. Shim et al. [58] fabricated the PCL/PLGA biodegradable 3D scaffold to deliver antibiotics to cure the chronic osteomyelitis. Some natural and synthetic biocompatible bone substitutes have been developed for 3D printing applications to promote bone regeneration as alternatives to autogenous or allogeneous bone grafts [59]. PLGA, calcium phosphate (CaP), TCP, collagen and chitosan, chitosan, HA, agarose, and gelatin have been chosen as 3D printing materials [37]. In particular, CaP ceramics appear to be particularly attractive materials for bone tissue engineering. CaP slurries or cement are typically extruded through a non-heated print head or nozzle under mild conditions, and the extruded materials are then solidified by a variety of ways based on their chemical composition [60]. The 3D printed CaP scaffolds have shown tissue regeneration in *in vivo* models, including rabbit calvarial defect [61], rabbit tibia defect and porcine maxillary defect [62], and femoral defects [63]. In another study, Williams et al. [64] designed PCL scaffolds with porous architecture and sufficient mechanical properties for bone tissue engineering applications. Seitz et al. [65] investigated the capabilities of a modified HA ceramic-based process chain for the fabrication of porous scaffolds for bone tissue engineering. A highly macroporous

3D bone structure was fabricated using an EPS. The 3D printed structures were effective as custom implants for bone replacement. Shim et al. [66] fabricated a PCL/PLGA/ β -TCP guided bone regeneration (GBR) membrane that slowly released rh-BMP-2 using 3D extrusion printing. In the rabbit calvarial defect model, the animals implanted with GBR membranes implanted group experienced improved bone healing after 8 weeks compared to untreated animals. In SLS fabrication, microhydroxyapatite, and nanosilica sol were utilized for fabrication of bone scaffold using a self-developed 3D printing apparatus. Bone scaffolds fabricated under the optimal manufacturing process possessed suitable biocompatibility and mechanical properties, permitting the cell adhesion and proliferation [67]. A polyvinyl alcohol (PVA) and HA composite were also used in SLS. HA particles were coated with a water soluble PVA via spray-drying or physical blending. These parts were used for craniofacial and joint defect applications. Studies both *in vitro* and *in vivo* have been conducted to examine the potential of printed bone scaffolds for bone regeneration. However, complete bone regeneration is typically not achieved without the addition of osteoinductive elements such as cells or growth factors [68]. There are still many unsolved problems concerning critical points related to the cytocompatibility and osteoconductivity of 3D printing technology [59]. The prospective work will be focused on searching the optimized combination of cells and other osteoinductive elements for the complete bone regeneration.

CRANIOFACIAL BONE

In this repair of bone, the morphological mimic is very important, so the method relying on 3D printing has long been studied actively. The 3D printing technology provides precise, fast, and inexpensive mandibular, skull bolt, and maxillary bone reconstruction, which aids in shortened operation time and easier surgical procedure. In particular, mandibular reconstruction can be challenging for the surgeon wishing to restore its unique geometry. Therefore, strategies are required to enable mimicking the 3D architecture. Recent advances in 3D printing have aimed to fabricate clinically implantable sized, and shaped bone grafts using synthetic materials. A custom-built 3D printer was designed to develop anatomically shaped PCL scaffolds with various internal structures. This study demonstrated the potential of 3D printed scaffolds to be used for engineering grafts for tissue regeneration [69]. Solid Freeform Fabrication technologies propose an enable fabrication method for the highly complex maxillofacial tissues [37]. 3D printing was also used to engineer scaffolds and regenerate the multi-tissue interface arrangement of bone and cartilage in the temporomandibular

joint (TMJ) [70]. In mandibular reconstruction, prefabricated 3D structure can help the surgeon plan precisely the plate and contouring aspects of the surgery. The 3D printing technology is a reliable method for precise mandibular reconstruction using bone plates and bone grafts [71]. In another study, indirect 3D printing has also been used to make customized implantable scaffolds for human mandibular reconstruction. Indirect printed gelatin molds shaped as the mandibular condyle were utilized to make porous PCL structures [72]. A 3D printed PCL plug fabricated by the FDM was utilized for healing alveolar bone. The implanted 3D printed PCL plug significantly enhanced bone healing in the fresh extraction sockets [73]. Kang et al. [11] presented the possibility of complex tissue or organ printing using an integrated tissue printing system focusing on the fabricating mandible and calvarial bone, cartilage, and skeletal muscle. Reichert et al. [74] examined custom-designed cylindrical 3D printed scaffolds designed to resemble cancellous bone in augmenting treatment of segmental bone defects of the tibia.

CHALLENGE OF CARTILAGE TISSUE PRINTING

Damaged cartilage tissue by osteoarthritis and joint injury is a primary cause of pain and disability [2]. In general, avascular cartilage cannot be recovered spontaneously. The general clinical interventions utilized for cartilage repair are microfracture, osteochondral graft, and autologous chondrocyte implantation [75]. Microfracture is the primary method used to obtain bone marrow stem cells for articular cartilage repair [76]. However, this invasive treatment cannot regenerate the hyaline cartilage [77]. Current cartilage tissue engineering trials also cannot fabricate native cartilage in terms of the zonal organization, extracellular matrix synthesis, and mechanical properties [78]. In addition, unsatisfactory mechanical properties have been obtained in clinical results of implanted or regenerated tissue [79]. Thus, 3D printing techniques are actively being applied to improve cartilage and disc tissue fabrications, as shown in Table 3. While conventional material processing techniques can be highly effective in scaffold engineering, the structures that comprise cartilaginous tissue in the craniofacial region, such as the interarticular disc in the TMJ and the auricular cartilage, are inherently complex due to their geometrical anatomies. Higher printing resolution is particularly important to fabricate the anatomical complex shapes of cartilage joint. Simultaneous photopolymerization during multi-layer printing is critical to maintain the printed cells at their initial deposited positions and the entire printed shapes. Furthermore, cell sources and biological cues should be optimized to increase the syner-

gistic stimulation for the chondrogenic differentiation of 3D printed cartilage scaffold. Therefore, fabrication of the cartilage complex 3D structures is required precisely adjusting the bioprinting parameters and components of the bioink to regenerate the damaged cartilage. The forthcoming efforts will be focused on demonstrating the feasibility of fabricating anatomic structures by delivering cells and scaffold materials to mimic the native cartilage.

ARTICULAR CARTILAGE

Thermoplastics, such as PCL or PLA, have been utilized in cartilage tissue engineering. Studies have shown the formation and synthesis of the cartilaginous matrix without any adverse tissue response [80,81]. 3D printed PLA scaffolds have already been applied in cartilage and intervertebral disc repair [82,83]. In the studies, extrusion bioprinting was used to fabricate the PCL and cell loaded alginate construct. Printed constructs exhibited improved cartilage formation [84]. Cui et al. [85] utilized inkjet printing technology to fabricate native cartilage structures by targeted printing of chondrocytes and PEG hydrogel to precise positions. Cells in the printed neocartilage maintained a chondrogenic phenotype as determined by gene expression and biochemical analysis. A novel bioink composed of nanocellulose and alginate was also utilized to 3D print cartilage with high fidelity and stability [86]. Pati et al. [49] developed a 3D printing method for designing a cell laden structure with novel dECM bioink to provide a cell-optimized favorable microenvironment for cartilage tissue. Hong et al. [87] showed a tough hydrogel comprised of PEG and sodium alginate that could be used for cell encapsulation to apply to cartilage tissue printing. In another study, Rosenzweig et al. [88] printed acrylonitrile butadiene styrene and PLA scaffolds to provide mechanical stability for cartilage tissue engineering. This study showed that chondrocyte and nucleus pulposus cells grew well and produced ECM within the 3D printed scaffolds. Hybrid printing of inkjet printing and electrospinning have been used to fabricate mechanically and biologically improved 3D constructs for cartilage tissue engineering. Fabricated constructs showed a cartilage-like tissue formation in both *in vitro* and *in vivo* studies [25]. Yi et al. [89] also combined an electromagnetic field (EMF) to improve the 3D printed tissue differentiation by generating a composite construct composed of polymeric framework and cell encapsulate alginate gel. The results showed the promotion of cartilage formation under EMF stimulation on the 3D cell printing system. However, there is still a lack of *in vivo* studies that can confirm the hyaline cartilage regeneration and the long-term stability after 3D printed tissue implantation. 3D printing for the carti-

Table 3. 3D bioprinting application for bone tissue engineering

Bone		Printing method	Printing material	Ref. (year)
Calvarial bone		Integrated tissue PEG-GelMA e gelth com	PCL polymer and Pluronic F-127 with composite gel	Kang et al. (2016) [11]
Mandible bone
Mandible bone		Indirect 3D printing	Polycaprolactone and chitosan	Lee et al. (2013) [72]
Mandible bone		FDM	PCL	Temple et al. (2014) [69]
Bone		Thermal inkjet printing	Poly(ethylene glycol) dimethacrylate and HA	Gao et al. (2014) [44]
Lumbar cage		FDM	Polycarbonate	Serra et al. (2016) [30]
Alveolar		FDM	PCL	Goh et al. (2015) [73]
Bone		Inkjet printing	Photocrosslinked PEG-GelMA	Gao et al. (2015) [45]
Bone (tibia segmental)		FDM	Poly(L-lactide-co-D,L-lactide) -TCP-PCL	Reichert et al. (2011) [74]
Bone		Extrusion plotting	HA	Seitz et al. (2005) [65]
Bone		SLS	HA and silica sol	Liu (2014) [67]
Bone regeneration membrane		Multi extrusion plotting	PCL/PLGA/ β -TCP	Shim et al. (2014) [66]

3D: three-dimensional, FDM: fused deposition modeling, SLS: selective laser sintering, PCL: poly(ϵ -caprolactone), PLGA: poly(lactic-co-glycolic acid), TCP: tricalcium phosphate, HA: hydroxyapatite, PEG: poly(ethylene glycol), GelMA: gelatin methacrylate, β -TCP: β -tricalcium phosphate

lage repair has a huge technical barrier to overcome. First of all, the choice of materials is very fussy. Sufficient physical strength has to be maintained during the time when the bio-material is being replaced with cartilage matrix produced by cells, and after the cartilage is repaired, the material has to be naturally and utterly degraded. Also, having a very smooth surface, it should minimize the friction of the surface during the repair is being done and should not cause any damage to the opposite side cartilage during the movement. Choosing and developing the biological materials that satisfy these conditions is important, and further, the application of the imaging systems that can mimic the form of tissues minutely and the printer that has high resolution is necessary.

AURICULAR CARTILAGE

Auricular reconstruction presents a challenge to plastic surgeons. 3D printing is becoming more relevant in auricular cartilage tissue engineering. The acquired digital images of a pediatric patient's ear were utilized for ear shape printing. The study showed that the tissue-constructed ears were durable for up to three months and possessed mechanical properties comparable to that of native auricles. Researchers have shown the superiority of 3D printing tissues to conventionally fabricated tissues [90]. As other trials, Lee et al. [91] printed the 3D ear shape structure with PCL and alginate. The sacrificial layer process was utilized to achieve complex shape printing. They con-

Table 4. 3D bioprinting application for fabrication of bone and cartilage composite tissue

Cartilage/bone composite		Printing method	Printing material	Ref. (year)
Osteochondral graft		Extrusion plotting and directional freezing	Chitosan-alginate	Reed et al. (2016) [75]
Osteochondral tissue		SLA	PEG and β -TCP	Zhang et al. (2014) [98]
Osteochondral tissue		Extrusion plotting	PLGA/ECM and alginate	Yang et al. (2015) [99]
Osteochondral graft		Extrusion plotting	PCL frame with collagen, or hyaluronan gel	Shim et al. (2016) [100]
Osteochondral graft		FDM	Poly(ethylene glycol) diacrylate	Castro et al. (2015) [101]
Osteochondral tissue		FDM	Poly lactic acid	Holmes et al. (2015) [102]

3D: three-dimensional, SLA: stereolithography, PCL: poly(ϵ -caprolactone), FDM: fused deposition modeling, ECM: extracellular matrix, PEG: poly(ethylene glycol), β -TCP: β -tricalcium phosphate, PLGA: poly(lactic-co-glycolic acid)

firmed chondrocyte and adipocyte co-printed ear-shaped structures induced ear cartilage tissue. Mannoor et al. [92] have also applied 3D printing technology to cartilage fabrication using a chondrocyte-seeded alginate hydrogel matrix with an ear shape and a conductive electronic component capable of transmitting sound. Using a tissue printing system with PCL and alginate bioink, Park et al. [93] demonstrated that cell-printed scaffolds induced better auricular cartilage tissue regeneration compared to cell-seeded scaffolds group *in vivo*. Sun et al. [94] reported a 3D printed scaffold using silk/collagen natural biomaterials was more suitable for cartilage tissue engineering than use of the conventional freeze dried technique. Recently, clinical applications of 3D printed auricular prostheses have been reported for three patients with microtia [95]. Reiffel et al. [96] has developed high-fidelity, biocompatible, patient-specific tissue-engineered constructs for auricular reconstruction of pediatric microtia and other auricular deformities both biomechanical and histological.

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A construction of a biphasic scaffold has been researched for the regeneration of composite tissue such as in the TMJ. Sherwood et al. [97] have developed osteochondral composite scaffolds with D, L-PLGA/L-PLA for the cartilage region, and LPLGA/TCP for the bone region. Osteochondral tissue has been receiving increased attention in cartilage research since improved cartilage regeneration would provide better shock absorption in subchondral bone. For osteochondral defect repair, 3D printed osteochondral graft is a feasible strategy for osteochondral tissue engineering applications, as shown in Table 4. Zhang et al. [98] printed biphasic osteochondral composite scaffolds using biphasic PEG/ β -TCP composites fabricated by SLA. These biphasic composites have been implanted in the rabbit trochlear defect model. The results indicated improved repair of both cartilage and subchondral bone. Reed et al. [75] also developed an acellular scaffold for the native zones of articular cartilage and subchondral bone. The 3D printing and directional freezing methods fabricated scaffolds that significantly mimicked the articular cartilage zonal architecture. In another study, Yang et al. [99] proved the plotting feasibility and tissue formation of 3D plotted biphasic grafts using PLGA and either cECM or HA blended with alginate for the regeneration of osteochondral defects. The bio-printing system included a dual-printing system that utilized 3D printing techniques and a computer-aided modeling system capable of creating heterogeneous osteochondral grafts. Shim et al. [100] also printed a multilayered osteochondral structure using 3D bioprinting to apply in the knee joints of rabbits. The 3D plotting fabricated

the mimicked native 3D extracellular environment to achieve the superior mechanical properties of regenerated tissue [101]. In another study, the extrusion 3D printing method was applied to print PLA osteochondral scaffolds with satisfactory mechanical property and biocompatibility [102]. Biphasic composite scaffolds of PLA and HA were manufactured and used to obtain a stable interface between cartilage and subchondral bone [70].

LIMITS AND FUTURE PROSPECTS

Technologies for 3D printing of scaffolds for implantable hard tissue are becoming increasingly innovative, and 3D printing has become a useful tool for the fabrication of complex structures. Based on previous research, 3D printing has been proven to be capable of producing tissue constructs of bone and cartilage. Hard tissue printing remains an emerging and growing technology with significant potential. Despite the many advantages of 3D printing strategies, there remain challenges to the 3D printing process and the materials required. The issues of printing tissue substitutes of larger size and uniformity that will allow clinical application will need to be solved. Among the printing technologies, extrusion plotting is the most suitable techniques for rapidly and precisely fabricating 3D structures of tissue and organ shapes [103]. However, it has some limitations, including low resolution, effects of shear-stress on cell survival, and limited material selection. By optimizing process parameters such as bioink concentration, printing mode, nozzle diameter, and cell amount, these limitations can be overcome to some extent. In particular, tissue ECM based 3D printing will be highly beneficial for biomimetic tissue and organ printing if mechanically enhanced dECM bioink composites can be printed without supporting materials. We predict that tissue differentiated 3D printed tissues will be developed with enough strength to endure implantation. In the meantime, the key technology of upcoming 3D printing will be realized from direct *in situ* printing onto or in the patient's bone and cartilage defects. Advancement in printing speed and layer resolution will enable *in situ* printing to enhance tissue regeneration with a short recovery time.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

There are no animal experiments carried out for this article.

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