



Three-dimensional bioprinting for bone and cartilage transplantation

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Abstract: Three-dimensional (3D) printing technologies can now be applied to biocompatible materials, cells, and supporting components. This 3D bioprinting could allow printing of artificial organs. Although this potential is far from realized, and despite the constant technical changes, 3D bioprinting has already been valuable in orthopedic applications like bone and cartilage repair, with advances in 3D modeling of defective tissue, stem cell differentiation, bioprinting technology, and innovations in printing materials. In the present study, we reviewed the recent advances in 3D bioprinting, as well as a general procedure for 3D bioprinting for bone and cartilage transplantation. In addition, we review the applications that aim to replace damaged bone and cartilage tissues, and research of *in vitro* tissue culture models. Potential challenges are discussed based on the present limitations and technical perspective of 3D bioprinting for bone and cartilage transplantation.

Keywords: Three-dimensional (3D); 3D bioprinting; 3D printing; bone; cartilage; transplantation

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Introduction

Additive manufacturing, which is also termed three-dimensional (3D) printing technology, is driving innovations in various fields, including manufacturing, healthcare, engineering, art, service, and medicine (1). Recently, advances have made 3D printings using by biocompatible materials feasible, and tissue regeneration studies are currently focused on printing additive cells and supporting components into complicated 3D functional tissues (2). Especially, for clinical applications, 3D printing, as a novel manufacturing technique, is being applied mainly in orthopedics. Recently, 3D printing-based, patient-specific surgical and dental implants were approved by the United States Food and Drug Administration and Caritas Europa for clinical use (3).

3D bioprinting is considered a useful method in tissue engineering because of its effective control over scaffold

fabrication and cell distribution. The 3D printing technology was first mentioned at 1986 by C. Hull, who named it “stereolithography”. In his design, thin layers of some materials cured using ultraviolet (UV) light were sequentially were built layer-by-layer to form a 3D structure (1). This technology has been used to create resin molds for 3D scaffolds made from biomaterials. Water soluble systems allows the additive manufacturing of biological materials, including growth factors and peptides, into 3D structures that could be transplanted directly, with or without cells (4). The following step is 3D bioprinting procedure as a method of tissue engineering for additive manufacturing technology, materials engineering, and cell biology. 3D bioprinting could also be used to produce patient-specific medical devices, such as splints and stents (5).

For use in orthopedic surgery, 3D printing faces

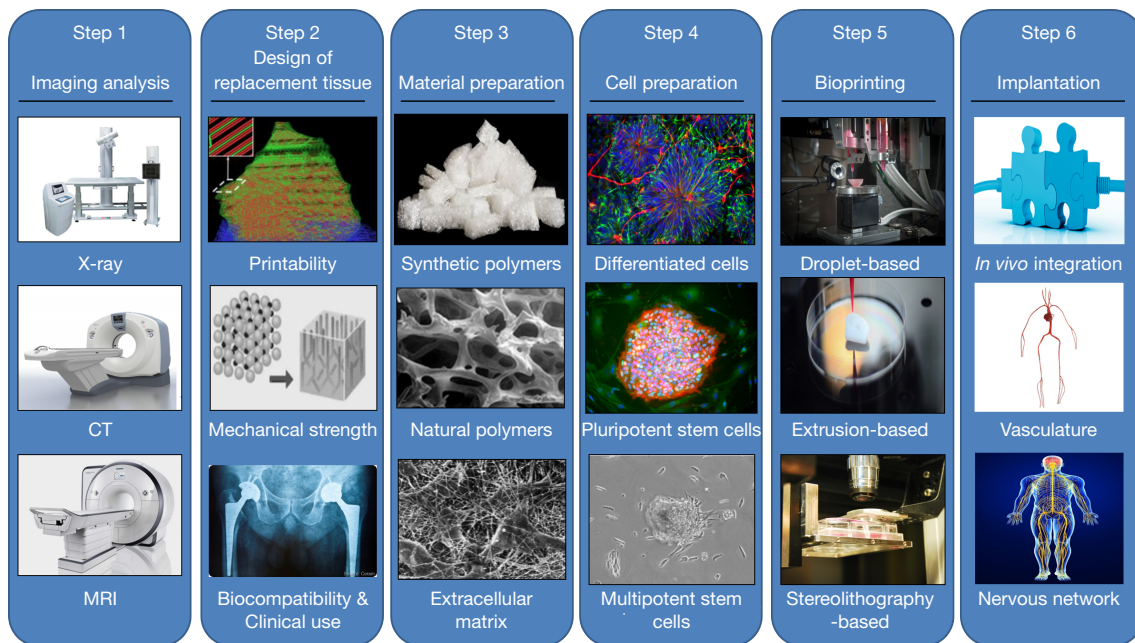


Figure 1 A typical 3D bioprinting process. The following steps are represented sequentially: imaging, design, material selection, cell selection, bioprinting, and implantation.

considerable challenges in cases of injuries that feature multiple bone fragmentation, as well as bone deformities. X-ray imaging is used routinely for orthopedic surgical planning. Yet, X-rays can provide inadequate information concerning the precise spatial lesion of bone defects (6). In spite of the advances in 3D anatomical reconstructions, the *ex vivo* 3D-printed product cannot perfectly match the defective lesion that needs to be regenerated. Current animal studies are based on an experimental bone defect model that can be filled with a scaffold that adopts the exact shape and dimensions (6). However, this is not the case clinically and although more defined defects can be generated (such as the ones made for mosaicplasty), this is not ideal as it further increases the area that needs to be repaired.

In addition, many studies have reported high molecular weight materials, such as poly-lactic-co-glycolic acid (PLGA), poly-lactic acid (PLA), and polycaprolactone (PCL), which are mixed with hydrogels to generate 3D printable materials that are compatible with cartilage component and cells (7). Although this research is rapidly growing, *in vivo* data of the capability of 3D printed material to reconstruct cartilage tissue are very limited (8). In particular, maintaining the long-term stability of the implanted tissue remains the major unresolved barrier.

Bioprinting 3D tissues typically involve imaging,

design, material selection, cell selection, bioprinting, and implantation steps (Figure 1) (1). Imaging of the defected lesion and its environmental situation can be used for design of 3D-bioprinted tissues. The choices of biomaterials and cells are essential and specific to the formation and function of the tissue or organ, and these components have to integrate with bioprinting systems. Some tissues and organs may require maturation through *in vitro* culture before transplantation.

Here, we review the typical procedure for 3D bioprinting of bone or cartilage for transplantation in orthopedic surgery. We also discuss a stepwise procedure for regenerating a complicated tissue, and present advances of 3D bioprinting technology for bone and cartilage regeneration.

Imaging analysis

The first step for 3D bioprinting is the generation of a medical image by the extraction of the target geometry from the patient. The imaging segmentation process make partition with an image area into non-overlapping connected regions that are homogeneous with respect to certain signal characteristics (9). Applicable noninvasive imaging modalities include magnetic resonance imaging (MRI) and computed tomography (CT). Computer-aided manufacturing (CAM)

and tools computer-aided design (CAD) are also used to gather and digitalize the complex tomographic information of tissues and organ (10). The 3D image is divided into horizontal or vertical slices that can be imported into the 3D printer system for additive deposition (11).

Main objective in the imaging process is to reduce the noise in the original image, leaving a comprehensive representation of the target characteristics. The difficulty of image partitioning depends on the degree of complexity. Image segmentation from diagnostic devices, such as MRI and CT, should end when the target lesion has been located. Actually, the image segmentation consists of assigning a label to each pixel, which is a component of a particular group (12). Classification using several segmentation algorithms has been described. All involve modifications of the anatomical analysis, therefore, the choice of the optimal way depends on the characteristics of the material characteristics (6).

A variety of commercial software enables medical image segmentation. The image segmentation procedure of each step starts from the appointment of a gray scale including all of the gray shades in target (13). The next step is to indicate one or more starting points in the images, which are curved surfaces where the algorithm will begin. The algorithm will develop within and through every slice till the user stops. If the structure or surfaces contrast well represented with its surroundings, the algorithm could be allowed to evolve until the region is identified without any further contrast (14). The processing depends on many parameters, including an expanded factor which can control the acting forces inwards and outwards on the imaging segmentation surface, and a curvature factor that can control how the 3D modeling surface loses its sphere shape and adapts to the detail 3D image (6). With complete algorithm process, the result is a 3D surface. The virtual 3D model can be navigable whenever and wherever a user interacts with it, with modification of the transparency or colors of 3D modeling, as are complex views of the interactions in the anatomical structures (15). The final process of imaging involves the transformation of the optimal format for subsequent 3D printing.

The global standard for 3D printing process is the standard triangulation language file format. This format represents a geometric of the target through the mesh, such as a series of triangles or honeycomb structures. Increasing numbers of oriented triangles generates an increasingly better surface quality object. In order to use in clinic, additional post-processing of the final 3D clinical imaging, such as removing noise, will be needed.

Design of replacement tissue

The raw data from the clinical image requires imaging modalities. Thus, the imaging process has to pass through tomographic reconstruction to generate cross-sectional images. In this process, the 3D design of replacement tissue from clinical imaging process can be produced for further modification (16). This process is described as the changes from an analytical anatomy to a synthetic anatomy (17). A method to produce 3D modeling of tissue structures is CAD-CAM and 3D modeling. This 3D modeling produces views of tissue structure while retaining the voxel information from clinical raw image that can be used for volume rendering and 3D imaging representation. Imaging reconstruction can be represented in various ways, such as wire-frame models, contour stacks, and solid and shaded model (18).

To generate an accurate design of artificial tissue from the imaging analysis, two-dimensional (2D) cross-sections and 3D representations could be applied for 3D bioprinting. Alternative speaking, a direct copy of patient's tissue may not be desirable and economically feasible. In these cases, data-based modeling may contribute to establish the structure of anatomical design (19). Additionally, computer modeling based on a patient's data can help predicting the biochemical and biomechanical properties of fabricated tissue manufacture (20,21). X-ray, CT, and MRI imaging have been used to provide accurate data of tissue dimensions and volume for the design of 3D printing constructs.

The 3D modeling of patient tissue is interfaced with 3D bioprinting systems through numerical control for prototyping. This method is established by reversing from 2D to 3D reconstruction of which the 3D modeling is divided into horizontal or vertical slices that are imported to the 3D bioprinting system. The structure containing the data of the 2D horizontal slices provides instructions for the layer by layer deposition (22). In addition, variations in the 3D bioprinting technique affects design of tissue. Some 3D bioprinting systems continuously deposit materials to form 3D structure. Another systems deposit two or three materials in extra spaces.

The 3D printing design has to be considered with four major principles for selection (23). Firstly, the biomaterials for 3D printing should have optimal properties to set the specific 3D bioprinter requirements, known as printability, which means the capability of the biomaterial to support manufacturing hardness. The interrelation of biomaterials and substrates must be investigated to accurate manufacture.

Secondly, biomaterials have to possess appropriate mechanical and physical characteristics, including wetting and swelling properties, degradation kinetics, internal and external structures ranging from the nano to macro scale, and structural stability. Thirdly, biological activity and biocompatibility must be considered the development and remodeling of tissues over long-term *in vivo* implantation. The biomaterial should provide engraftment with the endogenous tissue without any immune response, as well as a spectrum of biochemical cues such as growth factors, chemokines, and signaling proteins, which could affect cell motility, proliferation, and differentiation. Finally, the biomaterials should be affordable and readily available commercially with suitable regulations for clinical use.

Material preparation

3D bioprinting is an additive manufacturing process of laying-down successive materials, which include PLA, PLGA, and PCL, to fabricate physical objects from digital design, usually layer-by-layer, until the objects are created (24). In addition, ceramics like β -tricalcium phosphate (β -TCP) and hydroxyapatite (HA) are preferred for bone reconstruction. Bone graft substitutes are usually made from a composite of PLGA, PCL, and β -TCP. An effort to improve the biological function of 3D-printed synthetic scaffolds involves ornamentation with extracellular matrix (ECM) from the patient's tissue. The objective of tissue engineering is to lead to the repair and regenerate bone defects using a matrix scaffold as a platform to retain the indicated cells or therapeutic agents at the lesion site. The optimal scaffold has a similar complex of mechanical and biochemical properties as the native human tissue. To obtain these properties, construct must have a proper architecture favoring the flow of oxygen and nutrients for proliferation and differentiation of involved cells. It must possess osteoconductivity and be able to support cells through a suitable surface chemistry.

For cartilage bioprinting, the archiving of chondrocytes and stem cells encapsulated within alginate hydrogels is an attractive approach, since the cells retain their viability and metabolic activity (25). The main obstacle of the use of hydrogels for cartilage bioprinting is the difficulty in maintaining a uniform 3D structure. To avoid this problem, hydrogels should be mixed with synthetic biomaterials such as PGA, PCL, and HA (7). A combination of hydrogel, alginate-gelatin, and HA can be printed with a stable 3D architecture for bone and cartilage regeneration. This

mixture allows living human mesenchymal stem cells to be added to the biomaterials. This method has reportedly maintained cell viability after 3 days of *in vitro* culture despite the printing process and complete cross-linking synthesis (26).

Cell preparation

The 3D bioprinting technique accept the creation of novel constructs that lead to the precise deposition of cells to create an environment that is similar to native human tissue. The choice of cells for 3D bioprinting is critical for the proper function of the fabricated architecture. Whatever the specification of the 3D bioprinting method—whether the cells are combined with other biomaterials or are printed directly layer-by-layer on a construct or scaffold—the rate-limitation depends on the number of prepared cells (27). The optimum number of involved cells depends on the case of 3D bioprinter, and can range from 1×10^6 to 1×10^8 cells per mL (28). A large number of cells are required to synthesize the ECM support and enable the function of the construct. In addition, most tissues containing cells that provide supportive functions are involved in vascularization and nerve generation, or provide a niche for stem cell proliferation and differentiation. Current options of 3D bioprinting with cells involve either the deposition of multiple cell types into a complex that faithfully represents the native human tissue or the printing of stem cells that can proliferate and differentiate (29).

Cells for 3D bioprinting should possess several properties (30). The first is a sufficient number of cells that can be expanded during *in vitro* culture. The second is cells that are sufficiently robust to survive the printing process. The third is optimal properties of cell proliferation and differentiation in the scaffolds for either *in vitro* culture or *in vivo* implantation. The fourth is the ability to maintain cellular functions during *in vitro* culture and proliferate/differentiate after implantation by stimulation with the native surrounding, including growth factors or physical forces. The fifth is the structural and functional characteristics of the different cell types. The last is the interaction with multiple cell types for tissue development involving various biological signaling pathways.

The prolonged expansion and passage of cultured cells can result in the dedifferentiation of cells, and can affect cell physiology and genomic stability. Genomic and phenotypic changes as consequences of cell expansion lead to loss of cell function (31). Identification of the optimal cell type and functionality result in the successful production and

maintenance of a printed matrix as cells are specifically selected for their ability to synthesize a matrix that will offer integrity and robustness to architecture. Therefore, it is necessary to identify the cell type and functionality by investigation of properties like genomic state, phenotype, cell activity, and metabolism.

Bioprinting

3D bioprinting involves cells alive during the matrix fabrication process with the advantages of fast prototyping. Various printing technology has been developed to make up 3D networks of living tissue. Each has different restriction in terms of the available properties, which include printing speed, resolution, biological materials, and cell activity. The established technologies for 3D bioprinting used for deposition and patterning of involved materials are droplet-based, extrusion-based, and stereolithography. The different characteristics of these techniques have to be considered in light of the most critical factors in 3D bioprinting, which are cell viability, the involved materials and surface resolution (1,32).

Droplet-based bioprinting depends on diverse energy sources (electric, thermal, laser beam, acoustic, or pneumatic mechanisms) to deposit the microdroplets of biomaterial with living cells in high-throughput manners (33). Advantages are its simple characteristics and agility with precise control of biological components, including growth factors and cells for tissue regeneration. In addition, it is the most common method for pharmaceutical use because of its simplicity, versatility, and high-throughput capability.

Extrusion-based (direct writing or dispensing) 3D bioprinting, that originated from fused deposition printing, is used in mechanical, pneumatic, or electric systems to deposit cells and biomaterials using a “needle-syringe” format. During 3D bioprinting process, materials are precisely dispensed by the deposition system to print the cell-laden filaments and form the desired 3D architecture.

Stereolithography-based (vat photo-polymerization) 3D bioprinting employs laser beam to deposit the cell-laden biomaterial in a reservoir through beam scanning, allowing the molding of high-resolution patterns. Advantages include the precise control of the deposition of biological components and the very precise patterning.

Implantation

After the *in vitro* development of 3D architecture, the

manufactured tissue would be implanted into the patient's body for integration with another tissue or organ. This step involves immune acceptance, efficacy, and safety, and monitoring of the integrity and function of the 3D architecture after implantation. The most common and critical challenge is the integration with vascular and nervous networks in the 3D-printed architecture.

Endothelial cells and smooth muscle cells have been encapsulated in 3D bioprinted constructs without perfusable channels for the self-assembly of connected vascular (34). This process, which has been used to generate blood vessels in manufactured tissues, depends on the activities of endothelial cells to organize the blood vessels autonomously. Although designs of the implanted tissue lead to the formation of the vascular architecture, sometimes capillaries may be located too far from the arteriovenous loop, and the implanted tissue can be prone to necrotic process. In addition, from a 3D bioprinting standpoint, fabrication of the capillaries does not have to be considered, due to sprout from blood vessels based on endogenous angiogenesis, which is stepwise process of events that includes endothelial cell activation, migration, proliferation, and arrangement.

The human nervous system is widely distributed throughout the body. It controls the processes that coordinate voluntary actions, as well as the transfer of signals to different regions. Overall, regeneration of nervous networks means the involvement of a neural scaffold or conduit fabricated *in vitro* to the target site. The neural scaffold offers a direct framework for neurons to proliferate and secrete the inductive factors needed for axonal elongation. In the living body, neuronal cell resides within the 3D-ECM with micro- and nano-structures, and spatiotemporal physical and chemical cues. A conventional scaffold provides restrict control over geometry, especially for the oriented feature. The various 3D bioprinting techniques have allow more control over the internal structure and surface shape of the conduits, and has large potential for replicating the complicated nervous network.

Limitations of 3D bioprinting for clinical use

Bone and cartilage 3D bioprinting studies are exploding. Still, there is a paucity of *in vivo* data concerning the capability of 3D bioprinted constructs to regenerate bone and cartilage tissue. Especially, the long-term stability of the implanted tissue remains a formidable obstacle. Few studies have investigated the superiority of these techniques for clinical strategies. Thus, we are far from the clinical

use of 3D bioprinting constructs. One of the critical problems is the difficulty in gaining ethical approval for the harvesting of stem cells from patients and their expanded proliferation in the laboratory environment, as well as their subsequent use in surgery. This “bench-to-bedside” application of stem cells for therapeutic use remains more theoretical than real. There are obstacles to overcome before the discoveries made in the laboratory can be safely and successfully translated to clinical use (35,36). Another problem is the matching of a bench-based 3D bioprinted product to what is needed during surgery. In spite of the advances in 3D anatomic reconstructions, *in vitro* printed construct cannot perfectly match the lesion defect that is the focus of repair. Many recent studies have involved the creation of regular defects that are subsequently filled with a 3D printed scaffold having the exact desired shape and dimensions (36). However, in the clinical setting, although more defined defects can be created, this is not ideal, as it further increases the area that needs to be repaired.

Conclusions

Orthopedics and maxillofacial surgery were the first medical fields to use 3D printing technology. While there have been successes, the need remains for a printing system that can be used directly by the surgeon in real-time during the surgical procedure. This direct tissue engineering using 3D bioprinting techniques in bone and cartilage regeneration would avoid some of the laboratory-based manipulations that are now required. There are ethical and technical challenges to this goal. Moreover, using a single approach that avoids two or more surgical interventions would increase patient compliance and hasten recovery.

Although there are challenges to overcome for the use of 3D bioprinting in bone and cartilage regeneration, further multidisciplinary exploration and refinement of printing techniques, printable biomaterials, and 3D designs will hopefully realize the emerging potential of 3D tissue and organ bioprinting in the orthopedic and traumatology fields.

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