Mini-Review

BONE TISSUE BIOPRINTING FOR CRANIOFACIAL RECONSTRUCTION[1]

Pallab Datta[1], Veli Ozbolat 0000-0001-8328-4528[2,3,4], Bugra Ayan[2,3], Aman Dhawan[5], Ibrahim T. Ozbolat[2,3,6,7,2]

[1]Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology Shibpur, Howrah, 711103, West Bengal, India

[2]Engineering Science and Mechanics Department, Penn State University, University Park, PA, 16802, USA

[3]The Huck Institutes of the Life Sciences, Penn State University, University Park, PA, 16802, USA

[4]Mechanical Engineering Department, Ceyhan Engineering Faculty, Cukurova University, 01330 Adana, Turkey

[5]Orthopedics and Rehabilitation, Penn State University, Hershey, PA 17033, USA

[6]Biomedical Engineering, Penn State University, University Park, PA, 16802, USA

[7]Materials Research Institute, Penn State University, University Park, PA, 16802, USA

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[2]Corresponding Author: I.T. Ozbolat, W313 Millennium Science Complex, Penn State University, University Park, PA 16801Tel: 1(814)8635819, Fax: 1(814)8634358, E-mail: ito1@spu.edu

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Abstract

Craniofacial (CF) tissue is an architecturally complex tissue consisting of both bone and soft tissues with significant patient specific variations. Conditions of congenital abnormalities, tumor resection surgeries, and traumatic injuries of the CF skeleton can result in major deficits of bone tissue. Despite advances in surgical reconstruction techniques, management of CF osseous deficits remains a challenge. Due its inherent versatility, bioprinting offers a promising solution to address these issues. In this review, we present and analyze the current state of bioprinting of bone tissue and highlight how these techniques may be adapted to serve regenerative therapies for CF applications.

Keywords: Craniofacial tissue, bioprinting, bone repair, regenerative medicine

1. Introduction

The complex craniofacial (CF) anatomy includes a framework of bone, cartilage, muscles, skin and ligaments, supported by blood vessels and nerves. Congenital anomalies, cleft palate defects, traumatic injuries, infections, tumors and cysts, are the major etiologies of CF disease, which together account for a large number of deformities annually. Autologous vascularized free fibular flap has been the traditional gold standard for treatment of the majority of these pathologies. Limitations of volume and quality of donor tissue available and associated considerations of surgical time, cost, discomfort and associated post-surgical complications have driven efforts to search for better treatment options. These efforts include biomaterial and tissue engineering strategies to fabricate a load bearing matrix at the defect site while also providing a bioactive scaffold for cell adhesion, proliferation, differentiation and tissue formation. Allografts, prosthetic metals, and polymers have all being suggested as alternatives to autografts with varying clinical results (Visscher et al., 2016). Allografts may elicit an immunogenic response and have an inherent risk of disease transmission whereas the non-biologic materials demonstrate low wear resistance (metals), brittleness (calcium phosphates), and bacterial adhesion/infection risk (polymers). Previous experience has demonstrated that the fibular grafts and various biomaterials utilized for osseous reconstruction are unable to recapitulate the complex heterotypic

three-dimensional (3D) architectonics of bone within the CF region along with the location-specific heterocellular interactions, mechanical anisotropy, and necessary spatial patterning of growth factors (Nyberg et al., 2017). Controlling the dynamics of these processes will enable the development of predictable therapeutic modalities for treatment of CF injuries. Limitations of current solutions include the inability to customize variables in the defect size and surgical environments (*e.g.*, due to irradiation and infection). In the last decade, bioprinting has evolved as a scalable approach for fabrication of constructs.

Bioprinting is an additive manufacturing technique by which biological materials (i.e., cells) can be deposited precisely in accordance with a computer-generated pattern. Bioprinting offers unique advantages for direct, precise and on-demand patterning of multiple cell types and biomaterials as per a defined design, which can be obtained from the medical images of the defect area of each patient (Ozbolat et al., 2017), as schematically represented in **Figure 1**. The ability to directly print cells and extra-cellular matrix (ECM) components under physiological conditions allows for closer recapitulation of cell-cell and cell-matrix interactions found in vivo. To date, successful bioprinting has generated thin, non-vascularized tissues while fabrication of vascularized scalable tissue is in progress. Bioprinting of clinically-relevant tissues has been limited; however, *in-vivo* and *in-vitro* bioprinting studies demonstrate this to be a promising translational approach to address the complexities encountered in surgical reconstruction of CF tissues.

2. Image acquisition and bio-modelling:

A key advancement in clinical bioprinting of CF tissues is the integration of computer aided design (CAD) workstation, where medical images acquired by computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and ultrasonography. Acquired images can be directly fed into a processor, which can rapidly produce the design through image reconstruction algorithms. This step involves image segmentation and mesh generation followed by mesh optimization re-creating highly intricate irregular geometric models. The mesh is then converted into a path plan for bioprinting which can be directly fed into a bioprinter. These designs would be patient-specific with

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respect to not only the geometry of implant but also the level of tissue insufficiency and the anatomy of the composite tissue as well as the vascular network. Several advances in tissue segmentation algorithms from different imaging modalities have recently emerged that may eventually be used for bioprinting of composite vascularized tissues (Zhang and Chung, 2016).

3. Bioink Consideration

Bioprinting human bone tissue depends on the development of biocompatible inks which can be transformed from a flowable, liquid to a gel structure in conditions conducive to cell viability, proliferation, maturation and bioactivity. Results of investigations involving cell-based therapies, biomaterials and use of proteins, cytokines or growth factors (such as bone morphogenic proteins (BMPs)) in bulk scaffolds are useful resources for selecting bioinks for effective regeneration of CF defects. Bone marrow derived mesenchymal stem cells (BMSCs), adipose-derived mesenchymal stem cells (ADSCs) and chondrocytes have been the principal cell sources for the bioinks to date. BMSCs are multipotent cells that can be co-administered with clinically-available osteogenic cytokines such as BMP-2 and -7, fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), allowing more robust differentiation into osteoblast cells for bone growth. The inability to control the temporal and spatial effect of these cytokines and growth factors remains a challenge. Chemokine or ionic tethering and controlled release strategies are being developed to overcome some of these issues. Genetic engineering strategies to induce secretion of intracellular proteins resulting in successful bone healing can also be employed in bioprinting-aided regenerative medicine. The efficiency and safety of non-viral and viral vectors require more extensive standardization for large-scale clinical applications. It must be noted that cell selection is critical and should be determined with the size of the constructs required for regeneration in mind. The hydrogel component of the bioink is also crucial to the success of bioprinting. Commonly utilized hydrogel bioinks include collagen, alginate, gelatin methacrylate (GelMA) while ceramic bioinks composed of hydroxyapatite (HA) have also been explored (Hospodiuk et al., 2017). Nanoceramics can be utilized in bioink and mimic the role of HA in bone regeneration. The use of hydrogels is associated with several limitations including the lack of structural

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support to the printed construct as well as potential toxicity of the hydrogel crosslinking agents. Craniofacial bone is mechanically resilient tissue with both polymer and ceramic phases present in which HA crystals are responsible for providing the necessary strength. Composite scaffolds may better mimic these phasic mechanical properties. To date, bioprinting of bone has mostly employed scaffold-based approaches. Alginate and its crosslinker solutions are generally required for bioprinting to be co-extruded to allow for the ionic gelation. GelMA and other acrylated hydrogels like polyethylene glycoldiacrylate (PEGDA) can be easily crosslinked post bioprinting with UV radiation, but exposure to light may reduce cell viability. Combining polyethylene glycol (PEG) with GelMA as bioink renders mechanically more robust constructs while acrylated Arg-Gly-Asp (RGD) and acrylated matrix metalloproteinase (MMP) sensitive peptides can be added into the bioink to improve cell viability and reduce printhead clogging (Gao et al., 2015). For the purposes of bone bioprinting, scaffold-based approaches allow for more favorable initial mechanical properties of the printed construct. The polymer scaffolds and their hydrolytic breakdown products have been shown to be cytotoxic and may pose significant problems with biological incorporation, proliferation and growth beyond a specific period of time. Another advantage to the use of scaffolds in bioprinting is the ability to control gradient porosity properties, a property seen in native bone. Decellularized ECM has been used as a bioink carrier material for bioprinting various tissue constructs that allow cellular differentiation into specific lineages; to date, this has not been reported for bone. In addition to the cytotoxicity of the crosslinking mechanism, additional bioink considerations include compatibility with the selected bioprinting process, viscosity, the ability to mimic bone tissue properties, resolution, cost-effectiveness, scalability, mechanical integrity of the constructs, time required to achieve structural integrity post-bioprinting, maturation times, degradability, immunogenicity and involvement of multiple cell types (Hospodiuk et al., 2017).

When considering mechanical properties, it must be noted that human trabecular bone has an elastic modulus of 120-450 MPa in the mandibular condyle while in the region of mandibular midline to ramus these values are in the range of 112-910 MPa (Hollister et al., 2005). Hydrogels, on the other hand, have a modulus of 300-350 kPa indicating that mechanical properties of bioprinted constructs require

substantial improvement. In addition, several other bioink properties are vital for bone tissue bioprinting. These include optimized rheology (i.e., shear thinning and rheopectic properties for EBB and DBB, respectively) for bioprintability at cell densities that allow cell-cell interactions post-bioprinting and the ability to solidify rapidly under physiological conditions to maintain structural stability and shape fidelity of the constructs. A list of bioink materials used for CF applications is presented in Table 1.

4. Bioprinting process considerations

Bioprinting is performed with coordinated, controlled movement of a bioprinter, simultaneously depositing the bioink with living cells and utilizing one of three bioprinting modalities i.e. extrusion-based bioprinting (EBB), droplet-based bioprinting (DBB) or laser-assisted bioprinting (LBB) (Ozbolat et al., 2017). EBB involves pneumatic-driven or piston-driven extrusion of bioink through a nozzle while DBB employs electrostatic, thermal, piezoelectric, valve-based or acoustic actuation to deposit droplets of cell suspensions in a specific pattern. LBB exploits laser energy absorption by photoactive materials to transfer bioinks from a donor slide to a collector substrate according to the design. Both LBB and DBB are able to achieve very high resolution while EBB results in structurally and mechanically robust constructs. EBB is preferred for bone tissue bioprinting as it has the potential to create high initial mechanical strength constructs utilizing a range of bioinks. Using EBB, bioprinting can be integrated with the printing of mechanically robust polymeric frameworks, such as polycaprolactone (PCL) (see **Figure 2**) (Kang et al., 2016). In such constructs, the cell-laden hydrogel (Bioink) supports growth and proliferation of stem cells, and the PCL framework supports the structural integrity of the constructs. Other 3D printing techniques, such as electrohydrodynamic jet printing, can be used to potentially reduce the scaffold material volume in the construct, thus reducing the negative impact of slow degradation of PCL on bone tissue regeneration.

5. Bioreactor Considerations

Along with cells, scaffolds and growth factors, biophysical forces in the form of external stimuli (i.e. mechanical or biochemical), have become increasingly useful for successful fabrication of tissues. As discussed previously, the mechanical properties of bioprinted constructs, especially those printed using

DBB and LBB, are not well suited for initial load bearing; however, mechanical properties of the constructs can be improved after allowing the constructs to mature in bioreactors. A bioreactor can be defined as a vessel in which specific biological processes (i.e., tissue maturation) are accomplished under controlled operational conditions. Initially, bioreactors were employed for engineering scale-up constructs and ensuring proper mass transfer of nutrients leading to more homogenous distribution of cells. However, as the role of different external stimuli in directing cell differentiation became evident, bioreactors proved to be the most facile platform for exposing cells to such stimuli in a consistent and reproducible manner.

In a bioreactor culture, bioprinted cells deposit substantial ECM and improve the construct strength prior to implantation. Mechanical stimulation can also be applied which accelerates differentiation process by several mechanotransduction pathways (Rauh et al., 2011). Recent data shows that differential mechanical stimuli can lead to differentiation of stem cells into various lineages such as osteocytes, chondrocytes, etc. The mechanical stimuli can be applied in several ways including acoustic, fluidic shear or cyclic compressive loadings. For example, MSCs embedded within an alginate/collagen sponge will be induced to osteogenic and chondrogenic lineages when subjected to differential dynamic compressive loading (Michalopoulos et al., 2011). Moreover, several mechanical studies have revealed how fluid turbulence-induced shear stress stimulates osteoblast function through mediation of kinase-dependent pathways, intracellular Ca^{2+} release in vascular endothelial cells (ECs), wingless-family (WNT) signal transduction, and BMPs. In order to generate functional bone tissue to be used clinically, it is also necessary to consider certain factors that influence cell growth within bioprinted bone constructs. The most common bioreactors used for bone tissue engineering are the spinner flasks and rotating wall vessels, which rely on convection mechanism to increase mixing, as well as perfusion bioreactors (Sladkova and de Peppo, 2014). Range of shear stress in perfusion bioreactor are relatively low for driving robust osteogenesis but can be adapted for heterocellular cultures thus making them a system of choice for restorative applications in thin bone substitutes or bone patches (Rauh et al., 2011; Sladkova and de Peppo, 2014).

6. Recent achievements in bioprinting of bone tissue for CF reconstruction

Bioprinting using DBB: Several attempts have been made to engineer bone tissue for craniofacial reconstructions. Herberg et al. demonstrated the use of DBB technology to pattern growth factors (including BMP-2, the transforming growth factor-beta 1 (TGF-β1) and stromal cell-derived factor- 1 beta (SDF-1β)) on 5-mm-diameter acellular DermaMatrix (ADM) discs, which were then implanted into calvarial defects in mice (Herberg et al., 2014). Their results revealed that co-delivery of BMP-2 and SDF-1 resulted in significant bone regeneration compared to BMP-2-only and SDF- β -only groups (see **Figure 3**). In a series of studies to develop bone tissue from bioprinted MSCs, Gao et al have demonstrated the role of the bioink in bone formation (Gao et al., 2015). Bioactive ceramics have known osteoinduction properties and have been used clinically and surgically for bone regeneration for several decades. Bioactive glass and HA (in nanoparticle form) were co-printed with human BMSCs suspended in poly(ethylene glycol) dimethacrylate (PEGDMA). Solidification by photopolymerization after deposition ensured maintenance of high fidelity of the bioprinted constructs. MSCs co-printed with nHA produced scaffolds with Young's modulus values of 1–2 MPa and produced substantial bone tissue formation. Though the authors primarily focused on osteochondral joints regeneration, their results provide useful information for bioprinting constructs for CF region. Further improvements in osteogenic remodeling and biomineralization properties of bioprinted MSCs were obtained by combining collagen type I with thermo-responsive agarose hydrogels bioink. The organotypic combinations with high-collagen ratios can optimize mechanical stiffness required for differentiation as well as provide precise contours for the constructs (Duarte Campos et al., 2016). These studies represent significant advancements in bioprinting capabilities for creation of heterogeneous constructs for CF tissue engineering as such injuries often involve loss of skin, muscle or cartilage along with bone tissue.

Bioprinting using LBB: LBB was used to directly print nano-hydroxyapatite (nHA) biomaterials into cranial tissue defect sites in mice (Keriquel et al., 2010). Direct *in vivo* applications of 3D printing have been explored for formation of bone tissue; however, it was difficult to print mechanically strong, robust constructs. Similar to DBB, LBB also generates high resolution structures and is an orifice-free

technology minimizing shear stress-induced cell damage. However, this bioprinting modality has limitations for CF bioprinting due to its complicated set up and limited strength and thickness of the bioprinted constructs.

Bioprinting using EBB: Recently, Das et al. demonstrated osteogenic differentiation of human nasal inferior turbinate origin progenitor cells encapsulated in silk fibroin–gelatin, which was crosslinked either by the enzyme tyrosinase during bioprinting or by sonication post-bioprinting. EBB produced clinically-relevant (centimeter scale) analogues for tissue replacement (Das et al., 2015). Moreover, this work demonstrated that by changing the culture medium, progenitor cells could be differentiated into chondrocytes revealing the potential of this method to yield heterogeneous tissue constructs with differing mechanical properties and functions. In osteochondral tissue engineering, many CF defects such as cleft palate defects, periodontitis and edentulism would benefit from generation of tissue constructs. Extrusion-based bioprinting has been applied in coaxial deposition of mechanically-strong core-shell constructs made of calcium-deficient hydroxyapatite (CDHA) and alginate laden with pre-osteoblast MC3T3-E1 cells (see **Figures** 4A1-A4) (Raja and Yun, 2016). The composite tissue constructs retained high cell viability over time and reached a compressive modulus of 7.01 ± 0.82 MPa, whereas alginate-only constructs possessed a compressive modulus of 0.3 ± 0.03 MPa demonstrating the possibility of generating core-shell structures for CF bone regeneration. Bioprinting of alginate with multipotent stromal cells, calcium phosphate and plasmid-DNA (pDNA) encoding BMP has been also reported to form porous constructs (Loozen et al., 2013).This technique improved the efficiency of transduction of cells by pDNA, resulting in their differentiation as evidenced by BMP-2 and ALP production. This work demonstrated how bioprinting can be used in gene delivery-enhanced bone tissue formation as shown in **Figures** 4B1-B4. Despite *in vitro* bone formation, subcutaneous implantation of the constructs was not successful in inducing ectopic bone formation. The major drawbacks of this study resulted from the use of alginate as it does not readily undergo degradation *in vivo* and lacks inherent osteogenic or osteoinductive properties (Hospodiuk et al., 2017)**.**

Genetic engineering and gene therapy may improve long-term expression of osteogenic markers, as well as spatiotemporal control of this expression. There remain safety concerns with use of viral vectors for gene therapy as well as concerns with inadequate transfection efficiency with viral and non-viral vectors. Investigators have utilized sacrificial hydrogels to create porosity in the constructs, a key requirement for *in vivo* integration of bioprinted tissues as cell survival beyond 100–200 μm thickness is jeopardized due to limited diffusion of nutrients. The incorporation of micro-channels in bioprinted constructs is critical to allow access of nutrients to the cells (Kang et al., 2016). Key investigations demonstrate that such approaches can be particularly useful for CF defects involving damage to more than one tissue type. One of the concerns in EBB is that high shear forces can cause significant reduction in cell viabilities. However, EBB bioprinting offers numerous advantages for bioprinting of CF bone as a wide range of bioink (with high viscosities) can be processed, which is desirable for fabrication of mechanically-robust constructs.

Scaffold-free approaches obviate many of the limitations arising out of use of scaffolds including the biomaterial toxicity and their degradation products. Moreover, scaffold-free bioprinting allows for better cell-cell interactions, more robust deposition of ECM, as well as longer preservation of cell phenotype and function. Scaffold-free bioprinting has been illustrated using ADSCs and/or ECs bioprinted into multi-well plates with the NovoGen™ bioprinting platform, wherein osteopontin- and ALP-positive cells were observed after five days post bioprinting. Osteogenesis was confirmed by measuring several biochemical markers of bone remodeling including IL-1α, IL-6, IL-8, CCL2 and CXCL1 (Evinger AJ et al., 2013). A concern of a scaffold-free approach is the initial time-zero biomechanical strength of the constructs as compared to scaffold-based bioprinting.

Building upon this early success in bioprinting of thin bone substitutes or patches, ongoing efforts have been made toward the fabrication of scalable composite constructs containing muscle, skin, cartilage and nerves. Recently, successful bioprinting of composite tissues has been demonstrated using integrated bioprinting systems, which are capable of fabricating mandible bone with open channels (to facilitate vascularization and perfusion) and osteochondral tissue constructs (Kang et al., 2016).

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7. Future Outlook

Current bioprinting modalities are able to produce tissue constructs conforming to required geometry and architecture for reconstruction purposes. Mechanical properties of these constructs are still inferior to native bone and require more extensive investigations; thus, new bioinks and hybrid constructs with partial structural scaffolds are required. Hybrid bioprinting systems where the resolution, precision and viability of LBB or DBB can be combined with high mechanical strengths of EBB may also be a promising future direction. Strict standardization and quality control will be paramount to the process and progress. Tissue constructs can be fabricated in a facility within a clinical establishment under necessary surgical grade manufacturing practices and conditions; constructs can then be transferred directly to the surgery suite for implantation. Such a real-time modality containing imaging, bioprinting, operative planning and surgical execution can increase the therapeutic and logistical benefits as well as cost efficiency for CF regeneration. Advances made in bioprinting with respect to new methods and bioink formulations must be accompanied by considerations of clinical translation including the allied legal and regulatory frameworks. This will require investigators to address specific challenges of bioprinting compared to non-biological 3D printing, as it entails higher levels of material, cellular and technical complexities especially as related to the conditions required for living cells and their post bioprinting behaviors. In addition to bioprinting, 3D printing in medicine is still growing and evolving with advances and utilization in surgical models and guides as well as implants for CF reconstruction and dental applications. Specifically, 3D printing enables fabrication of drill guides, anatomical models and customized implants.

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Figure 1: Schematic representation of bioprinting process for CF bone regeneration. (A) Medical images can be suitably processed by algorithms to generate models of surgical regions required; (B1-B2) Medical imaging data can be converted to mesh files including soft and hard tissues (B1) mesh model was reproduced from National Institutes of Health 3D PrintExchange, (B2) was reproduced/adapted with permission from (Kang et al., 2016)); (C) Different bioprinting modalities used in biofabrication of bone tissue including extrusion-, droplet- and laser-based bioprinting (organ bioprinting concept images was reproduced from www.explainingthefuture.com); (D) the bioprinted constructs can be further matured in bioreactors; (E1-E2) Bioprinted constructs are then transplanted into patients ((E1) reproduced

from www.explainingthefuture.com, (E2) reproduced/adapted with permission from (Kang et al., 2016)).

Figure 2. 3D printing of supporting frame for fabrication of structurally stable hydrogel-laden constructs: (A1-A3) 3D printing of PCL frame with cell-laden hydrogels and fugitive hydrogel using an integrated organ printing system depicting osteogenic differentiation of human stem cells by Alizarin Red S staining (reproduced/adapted with permission from (Kang et al., 2016)); (B1-B2) An immunostaining image showing capillary formation in fibrin gel infused into PCL frame under vascular conditions on Day 14, CD31 (green, endothelial), αSMA (red, perivascular), NG2 (blue, perivascular) (scale bars represent 250 µm), (B3-B4) van Kossa staining of fabricated constructs showing mineral (black) deposition under osteogenic conditions (scale bars represent 100 µm) (images were reproduced/adapted with permission from (Temple et al., 2014).

Figure 3. The effect of bioprinting of different growth factor patterns, including (A1) control (no growth factor treatment), (A2) BMP-2, (A3) SDF-1β, (A4) BMP-2+ SDF-1β,on promoting bone regeneration on critical size cranial defects. (B1-B4) In-vitro osteogenic differentiation of MC3T3-E1 cells also showed differences in Alizarin Red staining (reproduced/adapted from (Herberg, 2014)).

Figure4. (A1-A3) Coaxial bioprinting of ceramic-polymercomposite structures for bone tissue engineering using CDHA and cell-laden alginate for the core and shell, respectively. (A4) Florescence microscopy revealed substantial number of live (green) cells (scale bars represent 1 mm) (reproduced/adapted with permission from (Raja and Yun, 2016)); (B1-B2) Macroscopic appearance of porous bioprinted constructs containing MSCs, ceramic particles with plasmid DNA encoding BMP-2 (scale bars in (B1) and (B2) correspond to 500 µm and 100 µm, respectively); (B3-B4) Osteogenic differentiation inside porous constructs (scale bar:100 µm) (reproduced/adapted with permission from (Loozen et al., 2013)).

Table 1: Different bioinks and bioprinters suitable for craniofacial reconstruction and their comparative analysis.

Bioink	Cell Type	Bioprinting Modality	Strengths and Limitations	Reference
BMP-2, TGF-β2, SDF-β1, Acellular Dermal Matrix	Acellular	DBB	Controlled osteogenesis due to growth factors; ability to pattern in high spatial resolution; inferior mechanical properties; amenability to print cell not studied.	(Herberg et al., 2014)
Nano-hydroxyapatite	Direct printing over defect site, no cells.	LBB	Osteoinductive bioink; ability to print in clinic; slurry compositions may be too viscous for processing of cell laden bioinks.	(Keriquel et al., 2010)
Silk fibroin, gelatin	Human nasal turbinate progenitor cells	EBB	Silk fibroin-EBB combination provides constructs with high strengths; Availability of the nasal turbinate cells in scale-up quantities may not be feasible.	(Das et al., 2015)
Collagen type 1, agarose	Mesenchymal stem cells	EBB	Collagen is a biomimetic material and a composite with agarose can improve the strength. Collagen bioink is expensive and difficult to isolate and process in cell friendly solvents.	(Duarte Campos et al., 2016)
Bioactive Glass, hydroxyapatite, PEGDMA	BMSC,	DBB	Osteogenic bioink is used but the mechanical strengths need improvement.	(Gao et al., 2015)
Calcium Deficient Hydroxyapatite, alginate	MC3T3-E1,	EBB	Osteogenic bioink with acceptable mechanical properties is used but MC3T3-E1 cells have toxicity concerns.	(Raja and Yun, 2016)
Calcium phosphate, pDNA-BMP	Multipotent stromal cells, pDNA encoding BMP.	EBB	The use of DNA bioink result in stable expressions but their toxicity concerns remain. Bioscaffolder printer requires to be more cost effective for clinical translation applications.	(Loozen et al., 2013)
Scaffold-free	ADSC, EC	EBB	Scaffold-free approach yielded limited time-zero mechanical strength.	(Evinger AJ et al., 2013)

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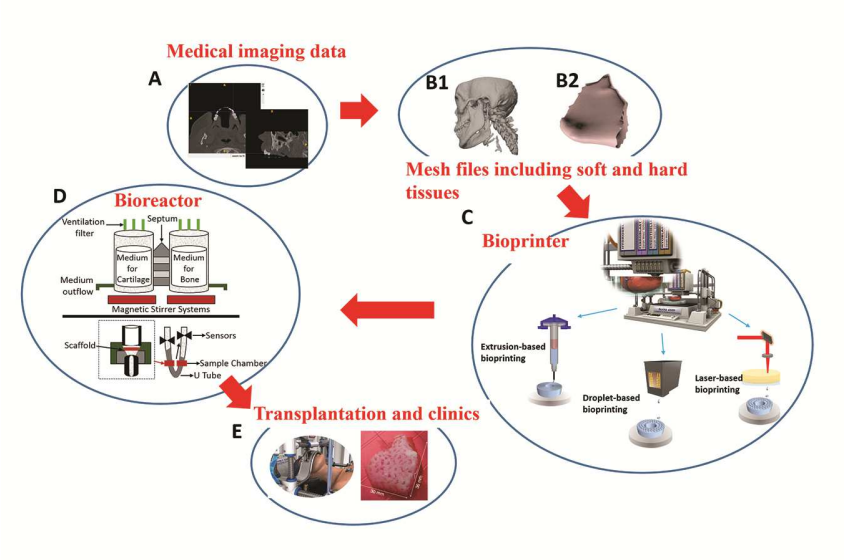


Figure 1

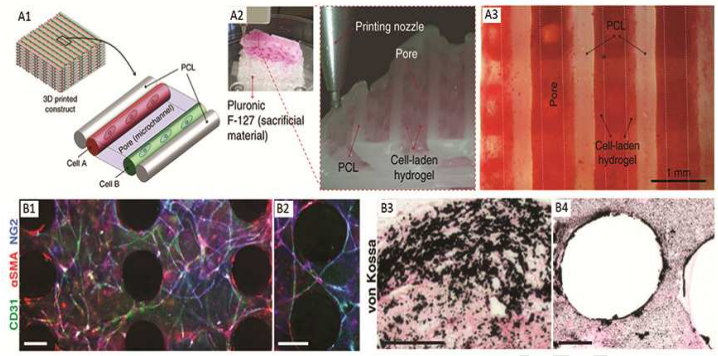


Figure 2

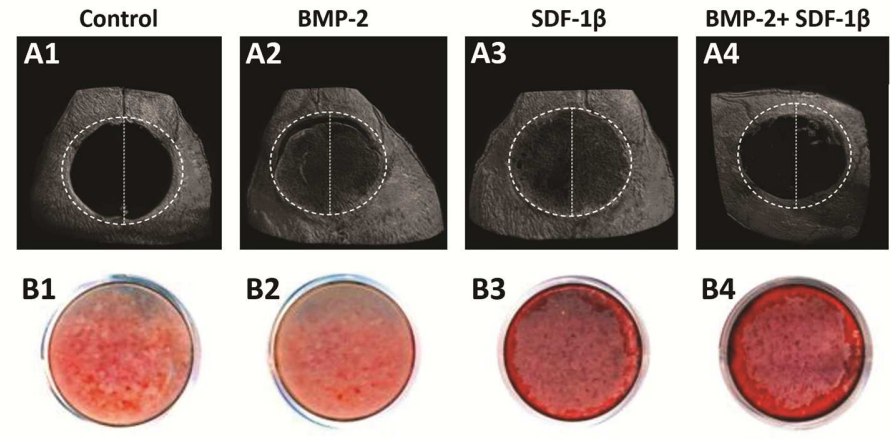


Figure 3

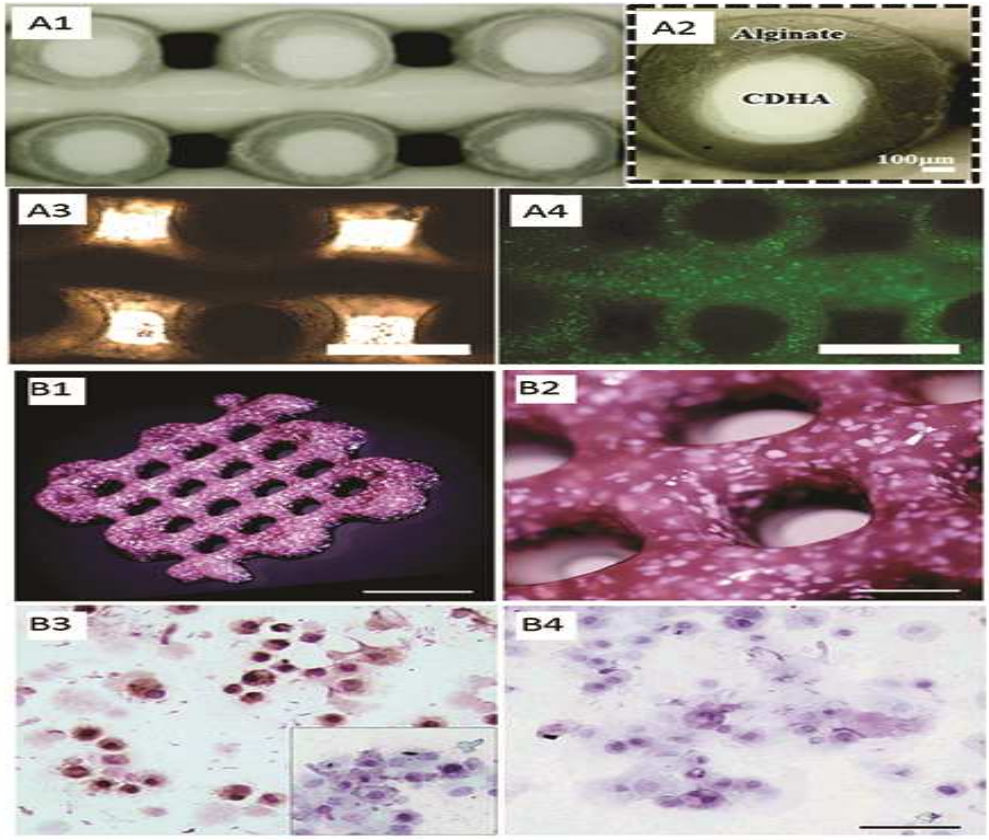


Figure 4

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