CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

Three-Dimensional Bioprinting for Regenerative Dentistry and Craniofacial Tissue Engineering

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Abstract: *Craniofacial tissues are organized with complex 3-dimensional (3D) architectures. Mimicking such 3D complexity and the multicellular interactions naturally occurring in craniofacial structures represents one of the greatest challenges in regenerative dentistry. Three-dimensional bioprinting of tissues and biological structures has been proposed as a promising alternative to address some of these key challenges. It enables precise manufacture of various biomaterials with complex 3D architectures, while being compatible with multiple cell sources and being customizable to patient-specific needs. This review describes different 3D bioprinting methods and summarizes how different classes of biomaterials (polymer hydrogels, ceramics, composites, and cell aggregates) may be used for 3D biomanufacturing of scaffolds, as well as craniofacial tissue analogs. While the fabrication of scaffolds upon which cells attach, migrate, and proliferate is already in use, printing of all the components that form a tissue (living cells and matrix materials together) to produce tissue constructs is still in its early stages. In summary, this review seeks to highlight some of the key advantages of 3D bioprinting technology for the regeneration of craniofacial structures. Additionally, it stimulates progress on the development of strategies that will promote the translation of craniofacial tissue engineering from the laboratory bench to the chair side.*

Keywords: 3D printing, biofabrication, bone regeneration, craniofacial regeneration, guided tissue regeneration, tissue scaffolds.

Introduction

Craniofacial regeneration strategies seek to mimic or promote oral developmental processes by using biomaterials and growth factors to induce tissue formation via stimulation of specific cellular function, both in vitro and in vivo. Craniofacial tissues, including bones, teeth, cartilage, muscles, and ligaments, as well as their fundamental building blocks, such as blood vessels and nerves, form complex systems responsible for a number of critical functions in the body. For instance, these structures work synergistically to ensure physiologic respiration, speech, digestion, and craniofacial support, among other specific roles. In nature, these tissues are organized with complex heterotypic 3-dimensional (3D) architectures, specific cell-cell interactions, anisotropic mechanical properties, and heterogeneous distribution of growth factors.

Because of the complex anatomy of craniofacial structures, full recovery of craniofacial tissues from trauma, resective surgeries, or congenital malformations is extremely challenging. Despite important recent advances in the field (Park et al. 2012; Ivanovski et al. 2014; Requicha et al. 2014), conventional regenerative strategies still largely fail to mimic the 3D complexity and the multicellular interactions occurring in native craniofacial tissues.

Three-dimensional printing of scaffolds, tissue analogs, and organs has been proposed as an exciting alternative to address some of these key challenges in regenerative medicine and dentistry (Derby 2012; Murphy and Atala 2014). This range of techniques, also referred to as solid freeform fabrication or additive biomanufacturing, enables precise positioning of cells and biomaterials in 3D with finely tuned internal and external architectures, while being customizable to patient-specific needs. Moreover, it allows for on-demand and scalable fabrication of complex designs, while being compatible with various scaffold materials and cell sources. Therefore, it represents a powerful approach for engineering

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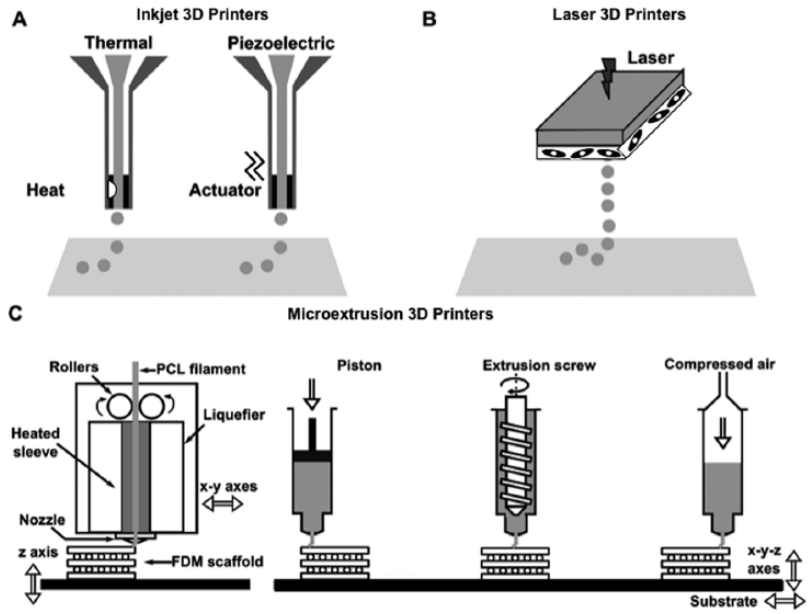
Figure 1.
Three-dimensional printing technologies. (**A**) Inkjet printing. (**B**) Laser printing. (**C**) Extrusion printing.



biomimetic craniofacial tissue constructs (Fedorovich et al. 2011). While more common 3D printing technology has been used to fabricate inert scaffolds for quite some time (here referred to as simply 3D printing), printing of all of the components that form a tissue, including living cells embedded in matrix materials (which here we refer to as bioprinting) is still in its early stages.

Here we review the application of both 3D printing and *bio*printing for oral and craniofacial regeneration. We describe different printing modalities and address their respective advantages. We then discuss material parameters associated with successful printing, and last, we review the current literature on applied strategies for craniofacial tissue regeneration.

Three-Dimensional Printing

Three-dimensional printing is a method that is fundamentally derived from additive manufacturing technology. In principle, objects are fabricated by adding materials layer by layer, hence rendering a 3D volumetric structure (Derby 2012). The printed structures are designed using computer-aided design (CAD) software or from images obtained via computed tomography (CT), magnetic resonance imaging, or X-ray. Traditionally, 3D printing has been primarily used to fabricate scaffolds constituted of synthetic inks (i.e., polymer hydrogels, sintered calcium and phosphate ceramics, inert metals), which are then seeded with living cells and tested in vivo after implantation. More recently, however, direct printing of living cells, cell-laden biomaterials, and scaffold-free cell aggregates also has been increasingly studied with a much greater level of complexity.

Although a wide range of commercial printers is available in the market and reported in the literature, most current systems fall under 1 of 3 categories: inkjet printers, laser-based printers, or microextrusion 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.

Inkjet 3D Printing

In inkjet 3D printers, volumes of liquid or low-viscosity inks, such as hydrogels or cell slurries, are delivered to predefined locations through diverse ejection procedures. Ejection is generated using acoustic, thermal, or electromagnetic forces. In continuous inkjet printing, a stream of fluid is passed through a small orifice in the print head, which breaks the fluids into small droplets (Saunders et al. 2008). In drop-on-demand (DOD) inkjet printing, the ink is dispensed in the form of droplets by applying a short pressure pulse through a nozzle, which varies from 20 to 50 microns in diameter. DOD can be divided according to the mechanism by which a drop is ejected. Thermal mechanisms are used to vaporize the liquid in a chamber immediately behind the printing orifice (Cui et al. 2012) (Fig. 1A). Alternatively, acoustic droplet generation methods produce a mechanical impulse that is applied by a rapid change in shape of a piezoelectric crystal behind the printing head (Fang et al. 2012) (Fig. 1A). In electromagnetic inkjet printers, on the other hand, an electrostatically driven mechanical displacement adjacent to a fluid-filled chamber (Xu et al. 2005) controls the droplet ejection.

Inkjet 3D printers have been extensively used to print living cells and tissue engineering constructs. Although most inkjet printers are compatible with high cell viability (Xu et al. 2005), the shear stresses caused by their extrusion through small orifices of the print head can be a limiting factor. Concerns relative to the material viscosity associated with frequent clogging have also been reported (Bajaj et al. 2014). Therefore, cells should be homogeneously distributed in low-viscosity inks or in the form of liquid slurries to prevent variations in the printing quality (Xu et al. 2005).

Laser-Assisted 3D Printing

Although laser printing is less common than inkjet and extrusion printing, its applications have been increasingly used for tissue regeneration. Typical laser-assisted systems used for cell printing have been developed based on laser-induced forward transfer (LIFT) technology (Guillotin et al. 2010). Laser printers basically constitute 3 main

components: a pulsed laser source, a transparent glass slide or "ribbon" (generally coated with an absorbing layer of metal) covered with a layer of cells and biomaterials of various viscosities such as polymer and dense cell solutions, and a receiving substrate (Guillotin et al. 2010) (Fig. 1B). In biological laser printing (BioLP), the energy of the laser beam is transferred directly to the bioink (Barron et al. 2004). In absorbing film-assisted (AFA-LIFT) printing, the laser is directed at an interlayer that transfers the energy to the bioink (Hopp et al. 2005). In matrix-assisted pulse laser evaporation–direct write (MAPLE-DW), on the other hand, the first layer of the liquid ink is vaporized, causing the ink to extrude (Ringeisen et al. 2004). A variation of common laser printers that has been used extensively for bone regeneration is the method known as selective laser sintering (SLS) (Peltola et al. 2008). This technique uses a laser over a thin layer of polymer powder to elevate the local temperature, causing the polymer to melt and fuse into a well-defined structure, where sequenced layers of melted polymer result in a 3D construct. However, this method is not compatible with printing of living cells.

Laser-assisted methods have been reported to enable printing of various materials with a broad range of viscosities (Koch et al. 2010), thus overcoming a common limitation of both extrusion and inkjet printers. Similarly, it is believed that the laser pulse used for the ejection transfer of the bioink has negligible effects in postprinting cell viability with several types of cells (Koch et al. 2010). In addition, laser-controlled systems that enable 3D fabrication of photocrosslinkable polymers from the top-down, known as stereolithography, have been vastly used for tissue engineering applications (for a review, see Zorlutuna et al. 2012). This particular method falls beyond the scope of this review.

Extrusion 3D Printing

A great variety of extrusion printers are available in the literature. In fused deposition modeling (FDM) systems, for instance, a premade polymeric filament is inserted into a liquefier/nozzle head and used to create the extrusion forces resulting in the deposition of molten-state polymer struts onto a collector/substrate (Zein et al. 2002). In these cases, since high temperature melts and extrudes the ink, polymers of high density and stiffness can be used. FDM is highly reproducible, with a relatively moderate speed, which enables control over the major physical characteristics of the resulting scaffold, such as mechanical properties, porosity, and pore shape (Hutmacher et al. 2001). In other extrusion printers, inks of low viscosity, molten polymers, injectable shear-thinning biomaterials, or cellular aggregates are dispensed using either mechanical action or a pneumatic system (compressed air) (Khalil et al. 2005). A metallic piston pushing directly against the ink (Bertassoni, Cardoso, et al. 2014; Bertassoni, Cecconi, et al. 2014) or inside a syringe acting as a plunge are common examples (Fig. 1C). The mechanical properties of these inks can vary considerably, depending on their composition. Hydrogels tend to have relatively low viscosity when printed as prepolymers. Cell aggregates will depend on the extent of cell-cell interactions and extracellular matrix (ECM) secreted by the cells forming the aggregate. Prepolymerized inks are typically dispensed as solidified and stiffer gels. In addition, a recently developed technology, called direct melt electrospinning writing (MEW), where a polymer solution or a polymer melt is forced through a high electrical field (typically above 10 kV), enables the deposition of micrometric fibers with accurate 3D control when using programmable stages (Vaquette and Cooper-White 2011; Dalton et al. 2013).

Materials for 3D Printing of Scaffolds and Craniofacial Tissues

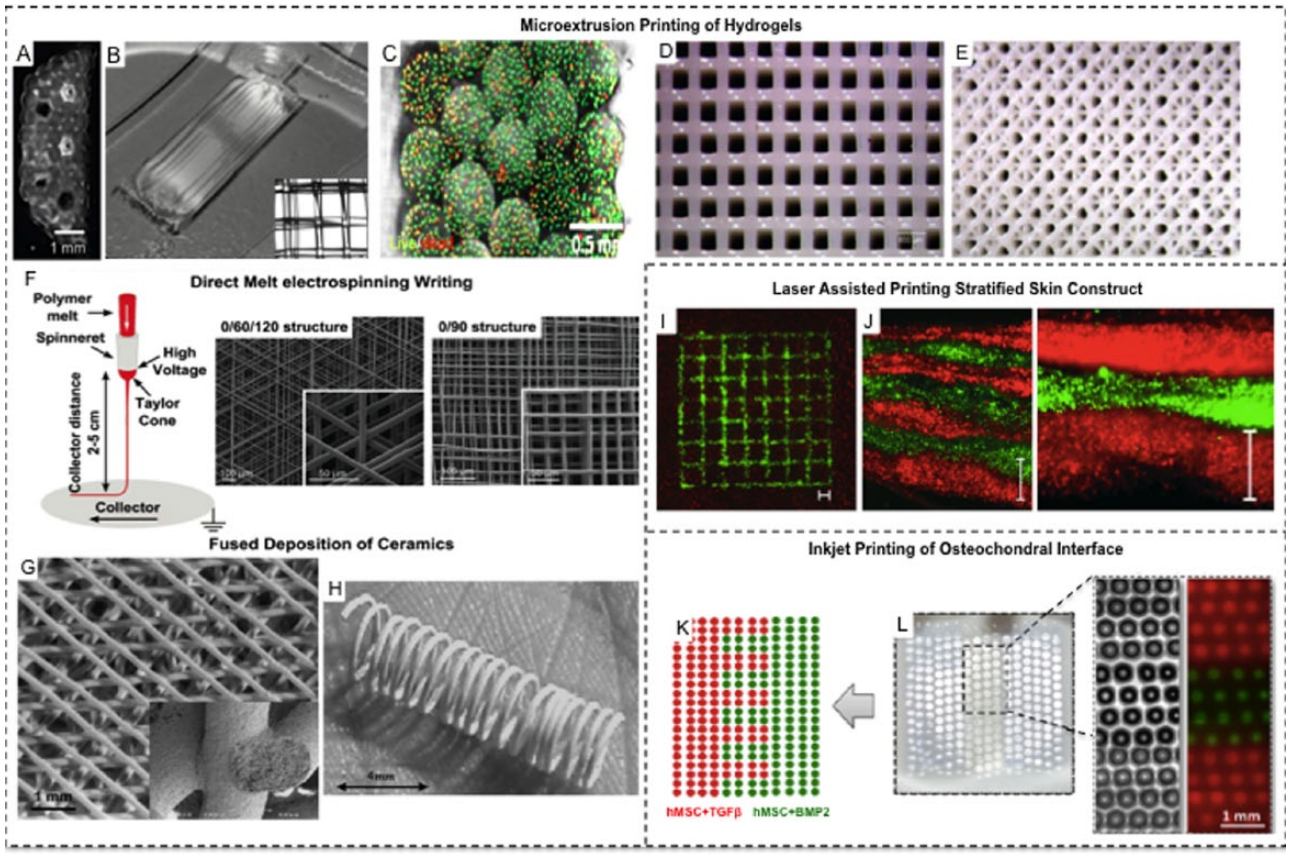
Polymers and Hydrogels

Polymer hydrogels are ideal candidates for the development of printable materials for tissue engineering. Hydrogels present remarkable tunability of rheological, mechanical, chemical, and biological properties; high biocompatibility; and similarity to native ECM (Annabi et al. 2014). Three-dimensional printing of polymers and hydrogels generally relies on the use of materials with controlled viscosity, which then defines the range of printability of the ink. Polymer inks, which are typically printed in the prepolymer phase, need to be viscous enough to allow for structural support of subsequent printed layers while being fluid enough to prevent nozzle clogging. To address the challenges of developing printable viscous inks, alginate hydrogels have been cross-linked with calcium ions immediately before the ink leaves the printing head or just after extrusions (Bakarich et al. 2014). More recently, prepolymerized cell-laden methacrylated gelatin hydrogels also have been used successfully for bioprinting applications (Bertassoni, Cardoso, et al. 2014) (Fig. 2A–C). Synthetic hydrogels used for cell encapsulation may limit cell-cell interactions that are required for efficient cell proliferation, differentiation, and tissue development. This can represent a limitation of bioprinting cell-laden hydrogels that is not present in 3D printed scaffolds with cells seeded onto or in bioprinting of dense cell aggregates, as described in "Cell Aggregates and Spheroids" below—hence the requirement for the development of ECM-derived hydrogels that have tunable physical and chemical properties, are compatible with high cell viability, and provide the adequate binding sites (RGDs) for cell attachment and matrix remodeling during their early proliferative stage (Annabi et al. 2014).

Synthetic polymers (Fig. 2D, E) are probably the class of materials most commonly used for 3D printing in biomedical applications (Woodruff and Hutmacher 2010). However, since high temperature is usually involved during the printing of these materials, the direct incorporation of cells or growth factors in the polymer mixture is generally avoided as the cell viability or bioactivity (Hutmacher et al. 2004) cannot be maintained throughout the manufacturing process.

Although hydrogels present a range of advantages for tissue engineering

Figure 2.
Three-dimensional (3D) scaffolds manufactured by different 3D printing methods for various applications. (**A–C**) Cell-laden methacrylated gelatin hydrogels printed with different architectures and high cell-viability. Adapted from Bertassoni, Cardoso, et al. (2014) with permission. Copyright 2014, IOP Publishing. (**D**, **E**) Printing of cell-free polymer filaments with orientations of 0/90° (D) and 0/45/90° (E). Adapted from Moroni et al. (2006) with permission. Copyright 2006, Elsevier. (**F**) Direct melt electrospinning writing, combining conventional melt electrospinning and additive manufacturing for the production of highly organized microfibrous scaffolds. Adapted from De Sousa et al. (2003) with permission. Copyright 2005, John Wiley & Sons. (**G**) Fused deposition of ceramics (FDC), a well-structured rapid prototyped scaffold after sintering. (**H**) Example of a complex structure manufactured by FDC. Adapted from Dalton et al. (2013) and Brown et al. (2011) with permission from RSC, copyright 2013 and John Wiley & Sons, copyright 2011. (**I**) Stratified skin-like architecture printed using a laser-assisted bioprinting system. Adapted from Koch et al. (2012) with permission. Copyright 2012, Mary Ann Liebert, Inc. (**K–L**) Interdigitated hydrogels and human mesenchymal cell cultures in the presence of transforming growth factor β and bone morphogenetic protein 2 for engineering of osteochondral tissue constructs. Adapted from Gurkan et al. (2014) with permission. Copyright 2014, ACS Publications.



applications, such as the ability of exposing cells to highly hydrated 3D microenvironments that closely resemble the natural ECM (Annabi et al. 2014), they generally present very low stiffness (in the kPa range) compared with the majority of load-bearing tissues that constitute the craniofacial region (in the GPa range). Therefore, printing of scaffolds for reconstruction of tissues subjected to higher mechanical loads, such as bones and teeth or the periodontal complex, usually requires the use of ceramic materials or composite scaffolds, where polymers are commonly combined with inorganic fillers to increase scaffold stiffness (Xavier et al. 2015).

Ceramics

Ceramic scaffolds are usually composed of calcium and phosphate mineral phases, such as hydroxyapatite (Michna et al. 2005) or β-tricalcium phosphate (Tarafder et al. 2014). Although ceramic scaffolds are not compatible with cell encapsulation for bioprinting, the ability of these scaffolds to upregulate osteogenesis due to the formation of a bioactive ion-rich cellular microenvironment, as well as their ability to mechanically provide space maintenance, makes these materials interesting alternatives for 3D scaffold fabrication for craniofacial applications. Moreover, in

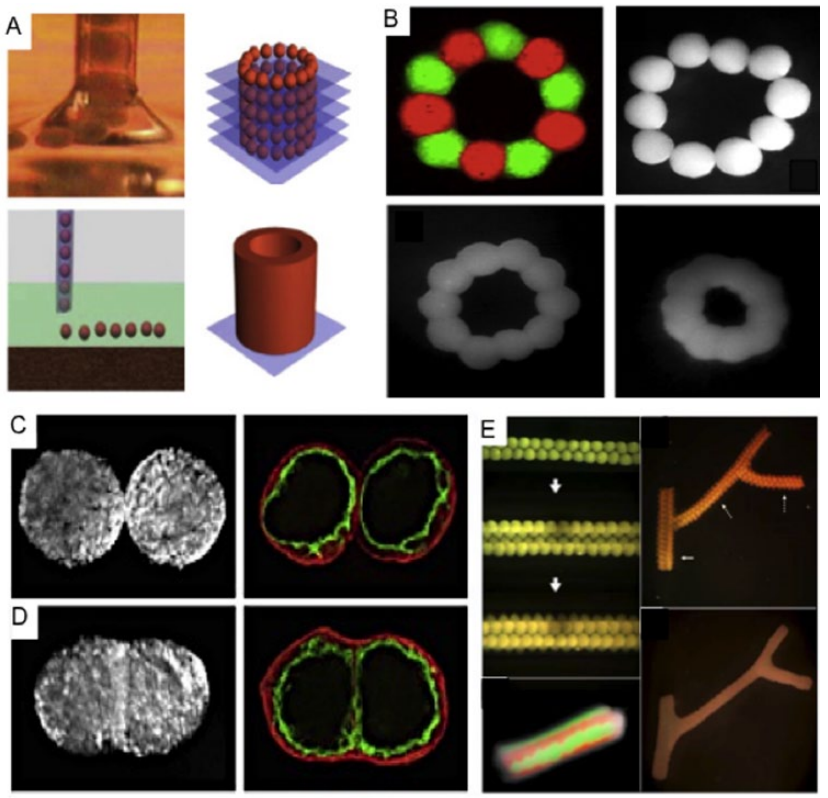
3D printed ceramic scaffolds, cells tend to quickly populate the scaffold surface, thus establishing close cell-cell interactions and promoting cell proliferation and differentiation. In addition, ceramics have much lower rates of degradation than hydrogels, which allows for prolonged guided tissue remodeling and structural support. Despite these advantages, ceramic scaffolds tend to be too brittle for implantation in load-bearing defect sites. Ideal scaffolds would combine the high calcium content of calcium and phosphate ceramics with the outstanding toughness of natural bone, which perhaps can only be obtained by creating scaffolds that are biomimetically mineralized and hierarchically structured, as recent attempts have shown (Wang et al. 2012).

Fused deposition of ceramics (FDC) in a direct printing mode generally consists of extruding a slurry including a high content (>50% w/v) of inorganic particles (De Sousa and Evans 2003) (Fig. 2G, H). The manufacturing of such scaffolds follows 3 steps:

1. Mixture phase, which involves the preparation of the slurry. The bioceramic particles are mixed in a solvent (aqueous or nonaqueous) with a low concentration of organic polymers/surfactants, called the binder, to obtain adequate flowability.
2. Green ceramic and binder burn-out phase involving the deposition of filaments of slurry following a predetermined pattern prior to drying and exposure to high temperature to burn out the organic component of the mixture.
3. Sintering phase, which involves the exposure of the green form to elevated temperature (above 1,000°C) to initiate the migration of atoms between adjacent ceramic particles, hence creating physical bonds called "necks."

Shape retention of the ceramic strut is critical for the reproducible manufacturing of 3D rapid prototyped bioceramics, a challenge that can be achieved by adjusting the viscosity of the slurry and the evaporation rate of the solvent (Morissette and Lewis 2000).

Figure 3.
3-dimensional (3D) bioprinting of the so-called "scaffold-free" tissue engineered constructs. (**A**) Example of extrusion printing of cell aggregates and schematic depiction of cell printing, 3D positioning of overlaid spheroids, and self-assembly of cell spheroids into an entire tubular construct. (**B**) Laterally positioned cell spheroids that maturate and assemble into a cell tissue ring. (**C**) Close microscopy view of just-printed cell spheroids and (**D**) self-assembled structure. From Mironov et al. (2009). Copyright 2009, Elsevier. (**E**) 3D printed long cellular tubes and branching vascular channels. From Norotte et al. (2009). Copyright 2009, Elsevier.



Composite Materials

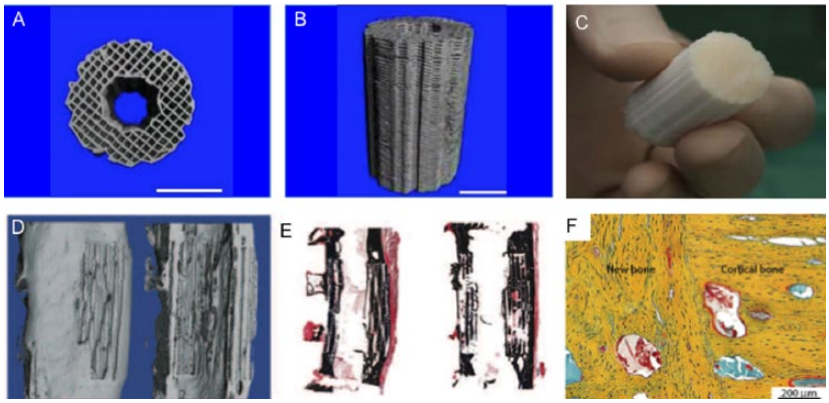
Printable composites, which are usually in the form of copolymers, polymer-polymer mixtures, or polymer-ceramic mixtures (Tevlin et al. 2014), allow for the combination of several advantageous properties of their respective constituents, thus forming interesting candidates for bioinks used in craniofacial regeneration.

In addition to the advantages of polymer composite hydrogels, such as interpenetrating polymer networks (IPNs) or hybrid hydrogels (Hutson et al. 2011), the incorporation of synthetic fillers to printable materials has also received extensive attention in the current literature (Bakarich et al. 2014). The addition of silicate fillers (Xavier et al. 2015) and a range of nanoparticles have been used to synthesize different types of composite scaffolds (Gao et al. 2014) to promote greater control over viscosity and stiffness of polymer hydrogels. More important, several of these silica-containing hydrogels show significantly higher expression of genes encoding morphogenetic cytokines, such as bone morphogenetic proteins (BMPs) (Müller et al. 2013). The combination of hydrogels with filler materials and/or natural peptides with morphogenetic capacity is certainly an area with great potential for future application in 3D printing for regenerative craniofacial repair.

Cell Aggregates and Spheroids

Three-dimensional bioprinting of cell aggregates and spheroids is a method

Figure 4.
Fused deposition modeling (FDM) medical-grade polycaprolactone–tricalcium phosphate scaffolds for regeneration of critical-sized bone defects (3 cm). (**A**) Three-dimensional (3D) micro–computed tomography reconstructions and a frontal section of an FDM scaffold after 3 mo and (**B**) 12 mo. (**C**) Safranin Orange/von Kossa–stained histology sections (orientation: top, proximal; left, medial) of regenerated bone after 3 and (**D**) 12 mo. (**E**) Movat's pentachrome-stained histologic sections showing the interface of cortical bone and newly formed bone (**F**) and a higher magnification image of the new bone. Reproduced from Reichert et al. (2012). Copyright 2012, AAAS.



that has received great attention since its development over a decade ago (Mironov et al. 2009). It is valid to point out that although this method is commonly referred to as "scaffold-free printing," generally small quantities of hydrogel are used to facilitate cell aggregation. In 3D printing of cell aggregates, multicellular spheroids are deposited using extrusion printers and allowed to self-assemble into the desired 3D structure (Fig. 3A–E). These systems allow for direct fabrication of tissue constructs with extremely high cell densities, which is a great alternative for solid, densely cell-populated organs. Its success for regeneration of load-bearing tissues, which are primarily constituted of proteins and minerals with relatively lower cell density, is yet to be demonstrated. Nevertheless, the ability to position aggregates of heterotypic cells with microscale precision may be an excellent alternative to bioprint pulp tissues with the stratified organization of odontoblasts lined by other cells types or to mimic the epithelial mesenchymal interface occurring in the early stages of the tooth organogenesis (Ikeda et al. 2009).

Three-Dimensional Printing Applied to Craniofacial Regeneration

Bone

FDM scaffolds were historically developed for bone and cartilage regeneration (Reichert et al. 2011) and hence are particularly suited for high load-bearing applications. Polycaprolactone FDM plugs were used for alveolar ridge preservation with some success as reported by Goh et al. (2015), providing an alternative to particulate synthetic calcium phosphate or deproteinized xenograft materials. In another recent example, cylindrical scaffolds of medical-grade polycaprolactone incorporating 20% of tricalcium phosphate microparticles were fabricated by FDM. Filaments of about 300 µm in diameter were positioned with a 0° to 90° pattern, separated by about 1,200 µm, thus resulting in a fully interconnected scaffold with 70% porosity and 22.2 MPa of elastic modulus. When these scaffolds were combined with recombinant human BMP 7 (rhBMP-7) and implanted in 10-cm critical-sized defects in a sheep model, which closely resemble human bone formation and structure, defect bridging was observed within 3 mo, and after 12 mo, significantly greater bone formation and superior mechanical strength were observed for the 3D printed scaffolds relative to the gold-standard bone autologous graft (Reichert et al. 2012) (Fig. 4A–F).

Periodontal Complex

Additive biomanufacturing technologies have recently been applied to the field of periodontal regeneration to develop hierarchical scaffolds, mimicking the properties and architectural configuration of the periodontium, which consists of both soft (gingiva, periodontal ligament) and hard (bone, cementum) tissues. These scaffolds are referred to as multiphasic constructs, as they possess various compartments recapitulating the native structure of the periodontal complex (Ivanovski et al. 2014) (Fig. 5A–C). One approach has involved the development of a biphasic scaffold based on the essential requirements for guided tissue regeneration (wound stabilization, space maintenance, and selective cell repopulation). It consisted of an FDM compartment to promote bone formation and a solution electrospun membrane, which was used in combination with cell sheet technology (Vaquette et al. 2012). This scaffold was further modified by depositing a layer of calcium phosphate onto the FDM bone compartment, and a larger pore size melt electrospun membrane was used to favor neovascularization of the periodontal compartment while enhancing the cross-communication between bone and periodontal ligament (Costa et al. 2014). These modifications significantly increased bone formation and permitted the attachment of functionally oriented periodontal ligament–like tissue.

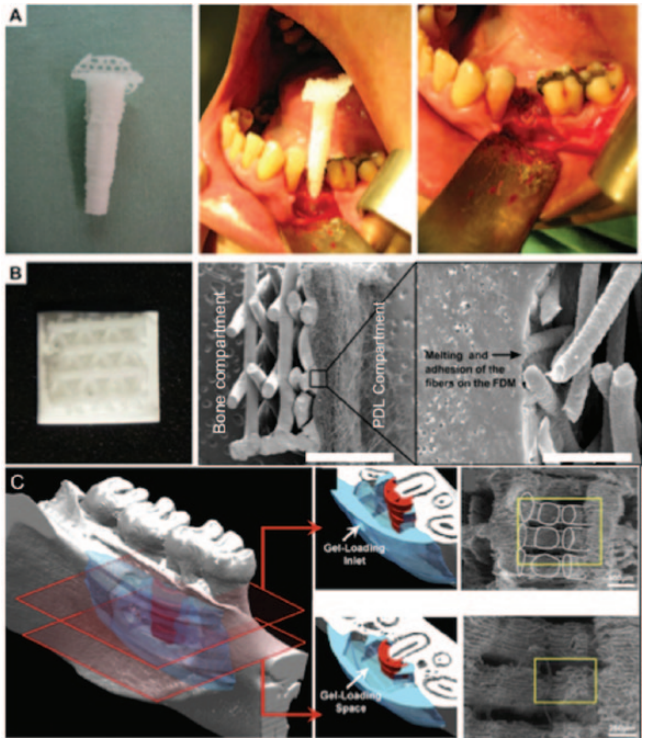
Park et al. (2010) used 3D printing to manufacture wax templates used in the fabrication of a biphasic construct. This template was designed according to specific architectural requirements (large pore size, perpendicularly oriented channels, and bone and periodontal compartments) and filled with a

polymer solution (Fig. 5A, B). A further advancement in this strategy involved the manufacturing of a customized biphasic construct obtained by CAD/computer-aided manufacturing (CAM) methodology, featuring aligned microchannels positioned along the surface of the periodontal compartment, resulting in a better controlled periodontal fiber orientation (Park et al. 2012). A recent study presented the development of a triphasic scaffold solely using 3D printing (Lee et al. 2014). This was achieved by adjusting the architecture (pore size, shape, and porosity) of the different compartments, resulting in highly compartmentalized, yet seamless scaffolds composed of regions that prompted growth of cementum-like tissue, periodontal ligament, and bone (Fig. 5C).

Cartilage

The structures that comprise cartilaginous tissue in the craniofacial region, such as the interarticular disc in the temporomandibular joint (TMJ) or the auricle cartilage, represent inherent complexity owing to their geometrical anatomies. Therefore, strategies that enable mimicking both the 3D architecture and the elastic properties of fibrocartilage are important developments in regenerative dentistry. A few noteworthy examples of 3D printing applied to cartilage repair have included the fabrication of a chondrocyte-seeded alginate hydrogel matrix with an ear shape and a conductive electronic component capable of transmitting sound (Mannoor et al. 2013). Three-dimensional printing was also used to engineer scaffolds and regenerate the multitissue interface arrangement of bone and cartilage in the TMJ (Schek et al. 2005). In this strategy, biphasic composite scaffolds of poly-L-lactic acid and hydroxyapatite were manufactured and seeded with fibroblasts, which were then transduced with adenovirus while expressing BMP-7 to stimulate cell differentiation into a chondrogenic lineage. Interestingly, the manufactured scaffold allowed growth of a stable interface between cartilage and subchondral bone, respectively (Schek et al. 2005).

Figure 5.
Fused deposition modeling (FDM) and 3-dimensional (3D) printing used for periodontal regeneration. (**A**) FDM scaffold specifically designed to match the anatomic dimension of an extraction socket. Adapted from Goh et al. (2015) with permission. Copyright 2015, John Wiley & Sons. (**B**) Biphasic scaffolds used in periodontal regeneration featuring an FDM scaffold fabricated for the bone compartment and an electrospun membrane used for delivering a periodontal fibroblast cell sheet. From Vaquette et al. (2012), copyright 2012, Elsevier and Costa et al. (2014), copyright 2014, John Wiley & Sons. (**C**) Rapid prototyping methodology used in an indirect printing approach. A polymer solution was cast into the sacrificial prototyped mold, creating a scaffold that allows for guided growth of periodontal fibers. Adapted from Park et al. (2012), copyright 2012, Elsevier.

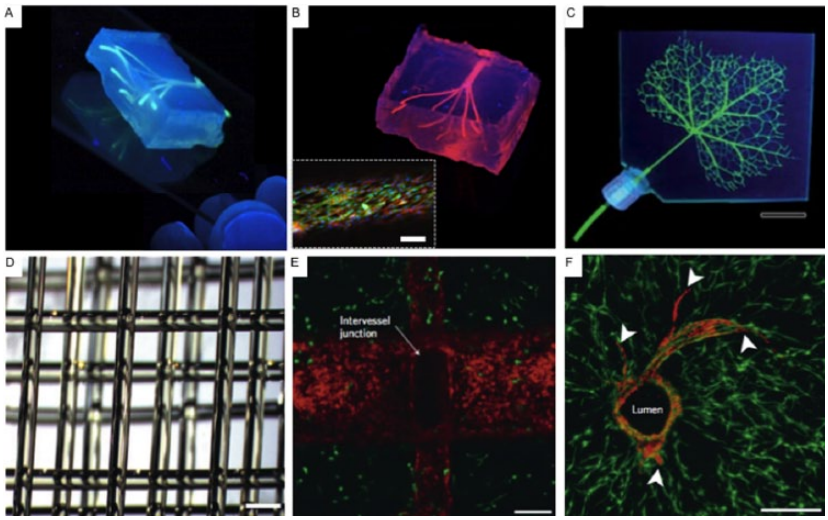


Pulp

Although direct examples of pulp regeneration via 3D bioprinting are, to our knowledge, still lacking in the literature, the fundamental building blocks of this tissue have already been successfully fabricated using different printing methods. In engineered cell-laden hydrogels, densely populated larger cell aggregates, and porous ceramic/polymeric printed scaffolds, the limiting factor for oxygen and nutrient delivery is primarily the diffusion properties inherent to different materials and the architecture at which they are organized. Therefore, supplying oxygen and nutrients for cells—which is efficiently performed by blood vessels and capillaries in the body—either in clinically relevant 3D constructs mimicking the pulp or any other tissue in the body has been one of the great challenges in regenerative medicine.

A few examples of biomimetic 3D printed functional blood capillaries have been recently reported by our group (Bertassoni, Cecconi, et al. 2014; Bertassoni 2015) (Fig. 6A, B) and others (Miller et al. 2012) (Fig. 6D–F). These networks are populated by endothelial cells in coculture with other cell types and have demonstrated effective delivery of oxygen and nutrients, as

Figure 6.
Examples of 3-dimensional (3D) printed vascularized constructs. (**A**) 3D printed branching agarose template fibers (500-μm fibers in green) inside cell-laden methacrylated gelatin hydrogels and (**B**) resulting microvascular networks perfused with a pink fluorescent die (inset). Formation of an endothelial monolayer inside the printed microchannels (inset: 200-μm scale bar). From Bertassoni, Cecconi, et al. (2014). Copyright 2014, RSC publication. (**C**) 3D printed closed-loop microvascular networks fabricated with fugitive ink and embedded in an epoxy construct. From Wu et al. (2010). Copyright 2010, RSC publication. (**D**) 3D printed degradable carbohydrate-glass templates (1-mm scale bar) and (**E**) resulting interconnected microvascular network coated with endothelial cells (200-μm scale bar) (**F**) branching into 10T1/2 cell-laden hydrogels (200-μm scale bar). From Miller et al. (2012). Copyright 2012, Nature Publishing Group.



well as waste removal from relatively large preosteoblast cell-laden hydrogels (Bertassoni, Cecconi, et al. 2014). Similarly, an interpenetrating hydrogel network of chemically cross-linked and physically entangled poly(2-hydroxyethyl methacrylate) hydrogel was 3D printed to guide the spread and proliferation of primary rat hippocampal neurons, hence forming differentiated, intricately branched engineered neuron networks (Shepherd et al. 2011). These examples, combined with recent developments in the extrusion printing of cell aggregates with controlled 3D architectures, represent great promises for the future of 3D printing of pulp.

Whole-Tooth Regeneration

Recent developments in the field of whole-tooth regeneration (Nakao et al. 2007; Ikeda et al. 2009) have shed light onto the potential of this technique for clinical applications. Early prototypes of 3D printed structures replicating the anatomy of the tooth and using composite inks of poly-ε-caprolactone and hydroxyapatite have already been tested in vitro and in vivo (Kim et al. 2010). The controlled formation of cell aggregates in vitro mimicking the epithelial mesenchymal interface that naturally occurs in the early stages of the tooth formation has been recognized as a fundamental step in whole-tooth regeneration (Nakao et al. 2007; Ikeda et al. 2009; Zhang et al. 2010). It has been hypothesized that architectural cues, such as size and positioning of cell aggregates, may be important determinants of the development and maturation of the tooth germ, as well as the positing and growth of cusps in the growing tooth. Therefore, the quest for controlled on-demand formation of engineered teeth may greatly benefit from 3D printing technologies where precise positioning of 3D cell aggregates is reproducibly achieved.

Conclusion and Future Directions

New advanced technologies under the banner of additive biomanufacturing allow the fabrication of structures closer in architecture to dental and craniofacial tissues. In their simplest form, this allows the fabrication of scaffolds upon which cells attach, migrate, proliferate, and ultimately form tissue-like buildups. A vast number of methods for 3D printing of scaffolds already have been successfully used for tissue engineering applications, but the 3D fabrication of fully formed and functional organs on the laboratory bench represents the next great challenge in the field of tissue engineering. A more exciting prospect is the printing and patterning in 3 dimensions of all the components that make up a tissue (cells and matrix materials) to generate tissue analog structures; this has been termed *bio*printing. It may be predicted that while bioprinting of less complex monolayered and hollow organs can be achieved in the foreseeable future, fabrication of functional solid organs will only become a clinical reality for future generations. In the meantime, it is only the effective interplay of engineering concepts in combination with the well-established fundamentals of biology that will realize the true potential of this exciting area.

Author Contributions

F. Obregon, L.E. Bertassoni, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; C. Vaquette, S. Ivanovski, D.W. Hutmacher, contributed to conception and data analysis, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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