

## ADVANCES IN 3D BIOPRINTING FOR BONY DEFECTS OF THE MANDIBLE

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### ABSTRACT

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**Background:** A series of conditions can leave the human mandible with a bony defect that is still difficult to compensate for with current clinical techniques. 3D bioprinting (computer-controlled, highly organized deposition of bio-materials and stem cells into a 3D structure) is a new tissue engineering strategy showing potential to contribute to the treatment of these defects.

**Objective:** The aim of this review is to give clinicians an idea of how 3D bioprinting works, where this technology is currently at and how it is developing towards clinical application in the field of maxillo-facial surgery.

**Data sources:** Bone tissue engineering literature was searched for articles that describe the use of additive manufacturing (collective term for layer-wise stacking of materials, including 3D printing) with use of biomaterials and stem cells.

**Study selection:** 3D bioprinting reviews and research articles presenting bone tissue constructs were selected.

**Data Extraction:** Information on 3D bioprinting background, design, applied techniques and used biomaterials for bone tissue were bundled. Research projects aiming at creating viable bone constructs were selected.

**Data Synthesis:** This review presents a comprehensive summary of 3D bioprinting basics and shows how this technique is evolving towards bone tissue constructs with the potential of clinical application in the management of bony mandibular defects.

**Keywords:** tissue engineering, 3D printing, bioprinting, biomaterials, bone, mandible.

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### 1. Introduction

Previous methods of dealing with substantial bony defects in the maxillo-facial area (most often arising from trauma, osteonecrosis, tumour removal or congenital disorders) have sought to replace the missing tissue with either artificial materials or with tissue from elsewhere in the body. To this idea, metals and polymers have been used, as well as autogenic parts of the fibula, radius bone, iliac bone and scapula. Though these modalities have proven themselves worthy of performing over not treating the defect at all, they both present some major downsides and limitations. Artificial materials remain foreign objects to the body, lack regenerative capacities and suffer from wear whilst autogenic transplants are never fully compatible with the defect, require difficult moulding and provide donor site morbidity.

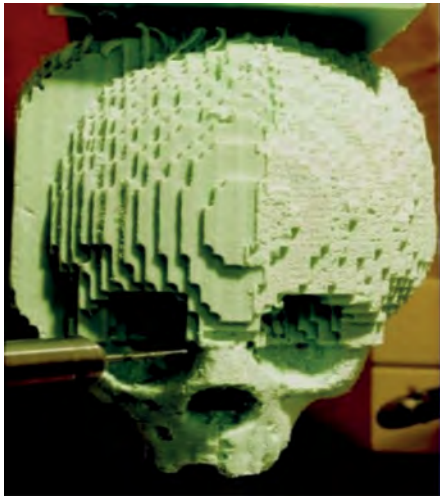
Recent advancements in the fields of stem cell research, biomaterial development and computerized 3D printing have given birth to a new tissue engineering strategy named 3D bio-printing, aiming to artificially recreate human tissue by cultivating stem cells applied onto (or even printed directly into) a 3D printed scaffold.

Most of the cutting-edge research that applies this tissue engineering strategy currently operates in the in vitro-domain, with just a few experiments transferring towards animal studies. Recent years however have brought incredible expansion of the spectrum of applicable materials and techniques. Researchers are creating extensive designs and customized processes to unite the properties of all of these techniques into constructs with a viability that was previously unknown outside of the body.

Following is an overview of the general workflow, the printing techniques, materials, properties and designs of 3D bioprinting and some illustrative examples of what is currently state of the art concerning bone tissue constructs.

### 2. Background

Paraphrasing the introduction, "3D bioprinting" is to simply put "3D printing with biocompatible materials and with living cells". It is thus a variant of 3D printing, a manufacturing technique which has been around since 1984, when Charles Hull invented an "Apparatus for making three-dimensional objects



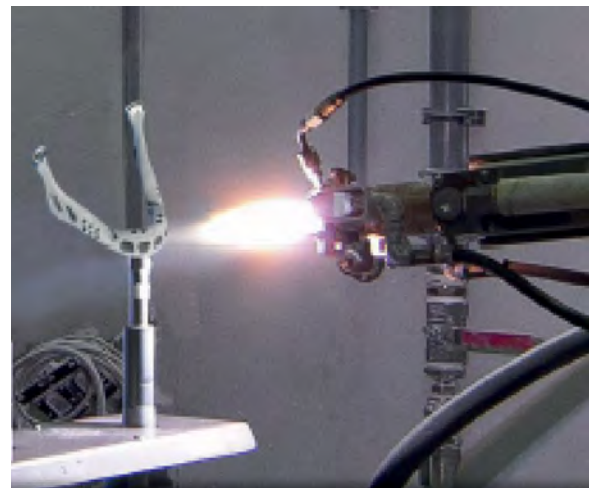
**Figure 1.** One of the earliest CT-based, plaster-milled study-models of a human skull (1987). Reprinted with permission by Dr. T. Lambrecht.)



**Figure 2.** An STL formatted model of a modern surgical wafer, printed by stereolithography[1]. Reprinted with permission by Elsevier.



**Figure 3**



**Figure 4**

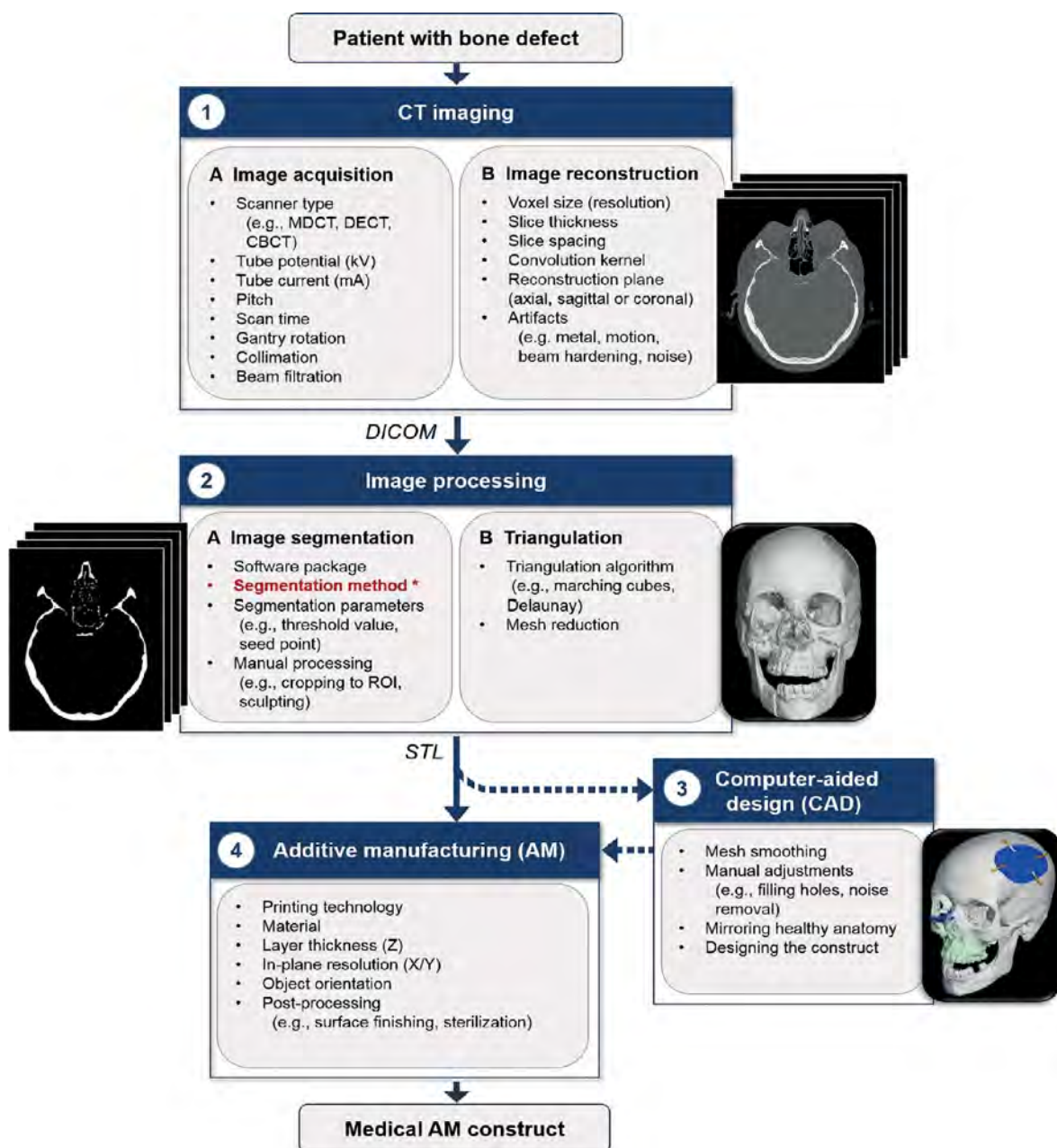


**Figure 5**

**Figure 3, 4, 5.** The hydroxy-apatite-coated, laser-melted, titanium full mandible, produced by Xilloc® and implanted by Dr Poukens in 2012. Reprinted with permission by Xilloc®.

by stereolithography". Steady development and improvement have made 3D printing applicable to several production processes in modern life and already useful in the maxillofacial clinic. For example, 3D printed study models (Fig. 1) have been around since 1987, proving themselves useful in evaluating pathology or planning of surgery. Several maxillofacial departments already use 3D printing to produce acrylic wafers [1] or cutting guides (Fig. 2), which are

then sterilized and applied during surgery. And (often in collaboration with specialized 3D printing design and production firms), patients are even treated with 3D printed, patient-specific implants, such as custom titanium meshes to cover up craniectomy sites and as of 2012 a fully functional, titanium mandible (Fig. 3), successfully implanted in an 83-year-old suffering from severe osteomyelitis.



**Figure 6.** Overview of the parameters affecting accuracy of medical AM constructs [5].

Overview of the process of creating personalized medical constructs by “additive manufacturing” (= collective term for production techniques based on layer-wise stacking of materials, including 3D printing methods) with parameters that can influence the accuracy of the constructs [5]. This research group found the differences between the imaged reality and 3D printed construct to range between 0.04 mm and 0.62 mm, when applying the most commonly used segmentation technique of “global thresholding”. Reprinted with permission by Elsevier.

### 3. From medical image to 3d (bio) printing design

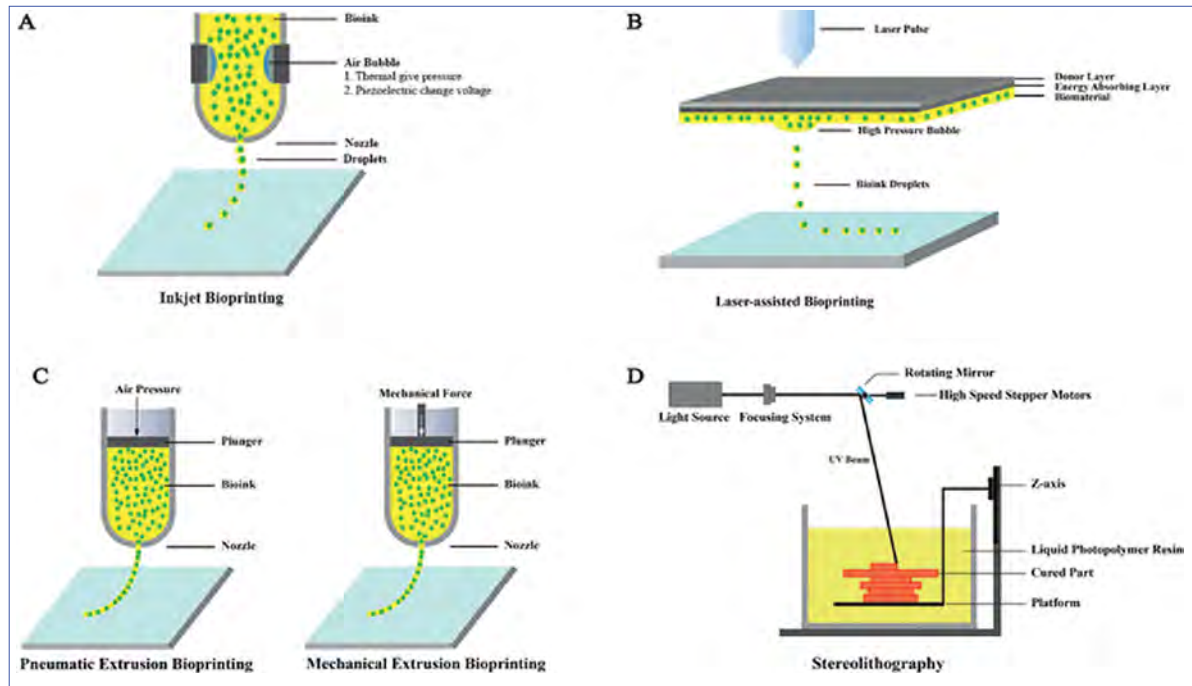
It is remarkable how early on medicine jumped on the wagon of 3D printing, with maxillofacial surgery apparently at its forefront. In part this reflects the great demand of maxillofacial surgery for highly customized constructs. On the other hand, the application of 3D printing in medicine has benefited greatly from the readily available high quality medical imaging, which can serve as a reference for the 3D printing construct (at least for the gross contouring) when processed by appropriate computer programs.

Converting medical images into a structural reference for the 3D printer usually begins with feeding the

medical images (JPEG, TIFF, BMP, but mostly raw DICOM data) through 3D converting software to create a 3D surface model [2]. This digital process performing this transition is named “segmentation” and the most popular type “global thresholding”. The model thus obtained can be a helpful tool by itself in evaluating existing pathology or planning surgery and can at this stage also easily be digitally adjusted, in which case it becomes a 3D CAD model.

A second process named “slicing” converts the obtained 3D surface model (or adjusted CAD model) into structural guidance for the 3D printer. So called “slicer-software” will firstly reconstruct the unstructured surface of the 3D surface model into a





**Figure 7.** Schematic drawing representing the major bioprinting techniques [12]. (A) Inkjet bioprinting (B) Laser-assisted bioprinting (C) Extrusion bioprinting (D) Stereolithography. Reprinted with permission by Wiley.

standard tessellation language (.stl formatted) model with a surface consisting of triangles (a process called “triangulation”, most often performed by applying the technique of “marching cubes”) [3]. Secondly the software will help design an appropriate structural framework to support the geometrically-approached surface and fill up the volume underneath.

After this, the software will effectively slice the obtained 3D model horizontally into layers and it will write “G-code” which will serve as computer commands for the 3D printer on the course it has to run the nozzle through while printing the digital model, layer on top of layer in a CNC-like way [4].

#### 4. The different types of 3D bioprinters

The previous paragraph described a design process that is very similar for 3D printers as it is for 3D bioprinters. The outside look of a ‘regular’ 3D printer and a 3D bioprinter can also be very similar, as companies such as ‘Cellink’ are now selling desktop models starting at approx. 5000 USD. However, most of the cutting-edge 3D bioprinting research performed nowadays makes use of very specific biomaterials and even more specific strategies of processing these materials. As a result, a lot of researchers modify existing 3D printers to these specific requirements, creating dozens of unique 3D bioprinters.

The exact method of delivering materials into a 3D structure however can always be narrowed down to one of 4 mechanisms, described below and illustrated in Fig.7 [6].

##### 4.1. Inkjet bioprinting

This type of bioprinting relies on the formation of small air bubbles to push droplets of bio-ink (the chosen mixture of biomaterials and cells) out of the nozzle of the printer. The bubbles can be generated by local heat (thermal), current over a crystal (piezoelectric),

sound waves (acoustic) or static electricity [7-11]. The first two of these methods are the most widely applied and even though heat generation would seem a risk for cell viability, more difficulty is experienced with the piezoelectric mechanism.

##### 4.2. Extrusion-based

Similar to inkjet bioprinting, extrusion-based bioprinting uses pressure to force the bioink out of the nozzle of the printer but does this by applying direct mechanical force or air pressure onto a plunger in a syringe-type of depositor [7-10]. With this robust depositing system, extrusion-based printers can handle more viscous types of bioink with higher cell densities (groups of cells, organoids), resulting in a continuous cylindrical stream rather than droplets.

##### 4.3. Laser-assisted

A third type of 3D bioprinting is based on the mechanism of laser-induced forward transfer of energy (LIFT). It uses the energy of a pulsed laser beam, focused and directed onto a specially designed 2-layer plate (called “a ribbon”), consisting of an absorbing layer generating local heat and ultimately small high-pressure bubbles which force droplets of bioink to form from an underlying plate of biomaterial [9,11]. Since there is no (mechanical stress-inducing) nozzle for the cells to pass through, cell viability is relatively high and precise focussing of the laser beam can provide good resolution of the printed construct. The process of printing however is rather slow and the 2-layered “ribbon” is an expensive component.

##### 4.4. Stereolithography

This last category of 3D bioprinting also applies focused UV light, but uses this to cure or selectively solidify a photosensitive biomaterial [9,13]. It is the oldest type of bioprinting and has yielded good resolutions with polymers with high molecular weights. The direct

Biomaterials used in 3D bioprinting	polymers	natural	<ul style="list-style-type: none"> <li>- collagen</li> <li>- gelatine</li> <li>- fibrin</li> <li>- hyaluronic acid</li> <li>- heparine</li> </ul> <p style="text-align: right;">Present in humans</p> <ul style="list-style-type: none"> <li>- alginate</li> <li>- chitosan</li> <li>- (carboxy-methyl) cellulose</li> <li>- pectine</li> <li>- silk fibre</li> <li>- chondrotine sulfaat</li> <li>- carrageenans</li> <li>- xanthan</li> <li>- dextran</li> </ul> <p style="text-align: right;">Found in bacteria, fungi,...</p>
		synthetic	<ul style="list-style-type: none"> <li>- PGA (poly-glycolic acid)</li> <li>- PLA (poly-lactic acid)</li> <li>- PCL (poly ε- caprolactone)</li> <li>- PPF (poly propylene fumarate)</li> <li>- PEG (poly ethylene glycol)</li> <li>- PU (polyether urethane)</li> <li>- PEEK (polyether ether ketone)</li> </ul>
	ceramics		<ul style="list-style-type: none"> <li>- Hydroxyapatite</li> <li>- B- tricalcium phosphate</li> <li>- coralline</li> <li>- Bioglass</li> <li>- calcium-silicate</li> </ul>
	composites		Hybrid hydrogels + ceramics, + synthetic polymer fibres + peptides + ...

**Table 1.** Overview of popular biomaterials used in bioinks.

UV lighting of the biomaterial however is known to induce stress, lowering cell viability.

### 5. Combining biomaterials and cells into the bioink

Up to this point we have described the part of 3D bioprinting that consists of computer programming and printing apparatuses. The part that is “bio”, consists of the cells that will be printed and the bio-compatible materials which will accommodate these cells. Together they form the bio-ink. To give an understanding of the spectrum of biomaterials, table 1 provides a summarization categorizing them as polymers, ceramics and composite materials [6,8,14,15].

The group of natural polymers consists of organic polysaccharides that are spontaneously formed by organisms in nature. Some of these appear in humans, such as collagen and gelatine. Others are of fungal or bacterial origin, such as chitosan [16] or alginate [17]. Since they attract lots of water, these polymers can be easily made into hydrogels, closely resembling

the natural cell environment and thus providing good bio-compatibility, osteo-conductivity and low immunogenicity [8]. These hydrogels can be printed at relatively low temperatures, which also favours cell survival. The lack of intrinsic strength however almost always demands crosslinking of the polymer; for example, with Ca<sup>2+</sup> or Mg<sup>2+</sup> in the case of alginate and NaOH in the case of chitosan. Crosslinking can be done by heat, chemicals or UV light, most often right after printing, but all of these are known to induce stress on the printed cells. When implanted in the body, the polymer construct would be degraded by enzymes such as collagenase and the degradation product would not be toxic. Variation in locale enzyme concentration however would make the degradation rate hard to control.

Using polymers that are instead synthetic linear aliphatic polyesters, would eliminate some of the problems of unpredictable characteristics associated with naturally occurring saccharides. Their molecular weight and size distribution are known and can stably be controlled and reproduced [14]. Their intrinsic strength is much higher than that of the natural



**Figure 8.** 3D printed gelatine mandibular condyle mold [24]. Reprinted with permission by Elsevier.



**Figure 9.** 3D-printed, porous PCL scaffolds of a mandible at 40% infill density [4]. Reprinted with permission by Wiley.

polymers; thus they allow constructs with a more complex architecture. Unfortunately, liquefying these synthetic polymers for printing requires temperatures between 60° and 200°C [18]. Degradation of these constructs, though spontaneous and predictable by simple hydrolysis, results in local accumulation of acids. Both of these characteristics negatively impact cell survival.

Bio-ceramics are also a category of biomaterials used in 3D bioprinting and they can rely on a longer tradition of clinical application, as some of them already exist as injectables and are approved by the FDA. They surpass synthetic polymers in compressive strength, and the often-high calcium content and porous microstructure provide good osteo-conductivity. However, as they are processed as a sludge, directly printing them together with cells is difficult and has not yet yielded high cell survival.

It is almost impossible to present an exhaustive list, as the category of composite materials is by far the largest as it is made up of combinations of materials. Not only combinations of components from the first two categories, but also combinations of biomaterials and bioactive compounds are currently in use. As mentioned above, most 3D bioprinting research is not performed by standard printers, but by custom-tailored variants. This is because researchers constantly try to unite biomaterial properties, printing techniques and tissue engineering designs into projects with viability. Some examples are described below. We have mentioned the good cell viability of natural polymer-based hydrogels such as alginate and gelatine, and their lack of intrinsic strength. Researchers went by this and have made mixtures of:

- Gelatine and acryl, resulting in methacrylated gelatin hydrogels [14].
- Alginate, gelatine and calcium phosphate, showing improved adhesion and cell proliferation when printed with bone-related Saos-2 cells [19].
- Alginate crosslinked with gelatine, combined with Bioglass [20] or Hydroxyapatite [21], showing improved mechanical strength, as well as improved proliferation and mineralisation when printed with Saos-2 cells.

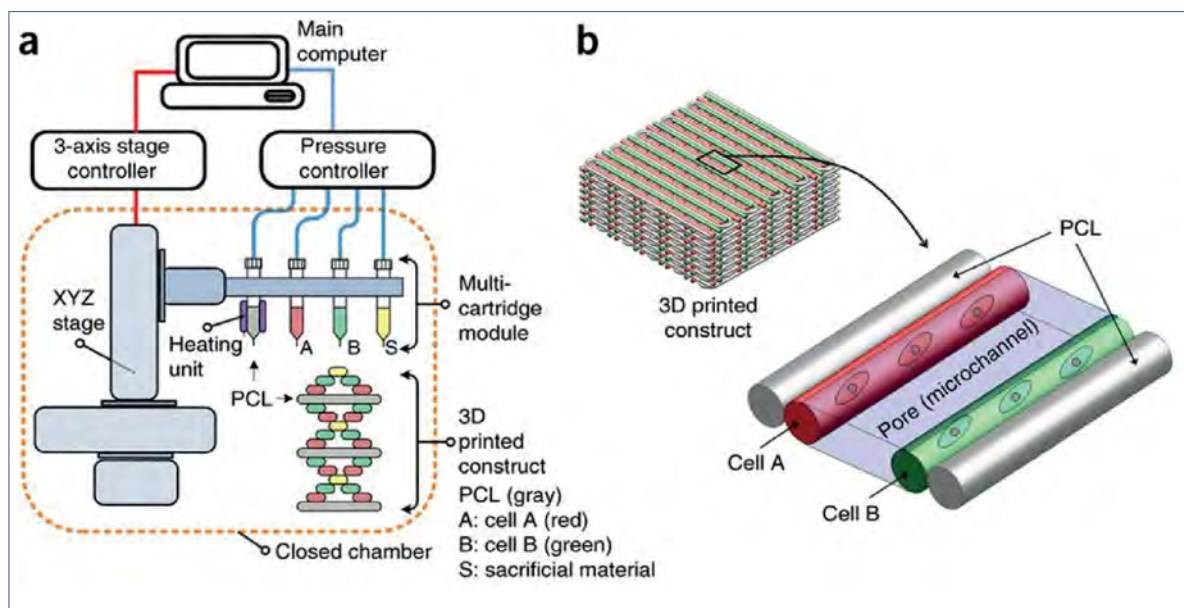
Synthetic polymers are generally stronger than natural polymers, but less bio-compatible. They too can be

made suitable for 3D bioprinting when mixed into composite materials, such as:

- Polycaprolactone crosslinked to alginate, resulting in improved strength [22].
- Polycaprolactone, added as a microfiber to hybrid hydrogels of alginate and gelatine, or to collagen, resulting in accelerated new bone formation, even in in vivo experiments [23].

These are just a few examples of biomaterial-configurations that show potential for 3D bioprinting, as they are able to unite viable conditions for living cells with processability and favourable characteristics towards scaffold design. Strictly, 3D bioprinting can be done by printing these combinations of biomaterials (without any cells) and allowing the printed construct to be colonised by cells, either in vitro or upon implantation. Some examples of this “indirect” 3D bioprinting are listed below.

In 2013 Lee et al. bioprinted a mandibular condyle out of gelatine (Fig. 8), with an outer surface and anatomical shape based on patient specific imaging and an inner structure of a regular (cuboid) lattice with tubes of 1.3 mm diameter and pores of 1.7 mm. By infiltrating the structure thus obtained with a PCL or chitosan solution and washing away the gelatine afterwards, the same construct was obtained in PCL and chitosan. These constructs were also successfully seeded with mouse bone marrow stromal cells (mBMSC’s), which showed good spread and proliferation, especially when coating the construct with hydroxyapatite [24]. In 2014, Temple et al. managed to bioprint a complete human mandible (Fig. 9) and maxilla directly out of PCL, using a self-designed 3D bio-printer (a converted CNC, able to melt and extrude PCL through a nozzle of 470 µm at a speed of 2.7 mm/s). They based the design on patient specific imaging and used a slicer programme which filled up the inner structure with a cuboid lattice and automatically created extra supporting structures, which were trimmed away after printing. The 3D bio-printed mandible however was not seeded with stem cells [4]. These examples demonstrate that reconstruction of a human mandible (or at least fragments) with an accurate shape and with some sort of viability has been lying within the interest field of 3D bioprinting research for some time already. Implanting these structures however would seem



**Figure 10.** The ITOP system [36]. (a) The ITOP system consists of three major units: (i) 3-axis stage/controller, (ii) dispensing module including multi-cartridge and pneumatic pressure controller and (iii) a closed acrylic chamber with temperature controller and humidifier. (b) Illustration of basic patterning of 3D architecture including multiple cell-laden hydrogels and supporting PCL polymer. Reprinted with permission by Nature America.

insensible, as we would all sense they would have to mimic the human mandible much closer if they should replace it, survive in its environment, be subject to the forces it withstands.

## 6. 3D bioprinting design

It is clear that more elaborate designs for 3D bioprinting are still to precede possible attempts at implantation. Executing these proceedings, many researchers are now focussing on creating smaller patches of tissue, with more realistic viability. Incredible ingenuity has already led to several successful constructs and in doing so, several 3D bioprinting parameters have been studied. The obtained insights are also broadening the view on how tissue engineering a load-bearing structure, such as required for bone tissue should be approached. A few key features contributing to a successful 3D bioprinting design for bone tissue are described below.

### 6.1. Configuration of the construct (design of the scaffold)

Current approaches towards load-bearing bone range from loose configurations of hydrogels already containing stem cells, to rigid, volumetric and morphologically adequate printed scaffolds, later to be seeded with stem cells. Generally, constructs are more bio-compatible and supporting of self-organising capacities of the cells as they are more natural polymer based, and more structured and mechanically strong as they contain more synthetic polymers or bio-ceramics. It must be noted that the majority of all 3D bioprinting research currently makes use of the more organised approach.

The scaffolds created are most often regular shaped, supported by space-filling lattices (regular cubes or honeycomb pattern (e.g. Fig 8)). This is despite the fact that algorithms and even libraries and tools have been developed to create more complex scaffolds with

functional grading and with known characteristics, more closely mimicking the complexity of tissue [25]. Supposedly it is due to the small scale of the research and focus on cell-viability that these more complex algorithms have not yet been applied to in vitro 3D bio-printing studies, because most likely they would not cause executional problems.

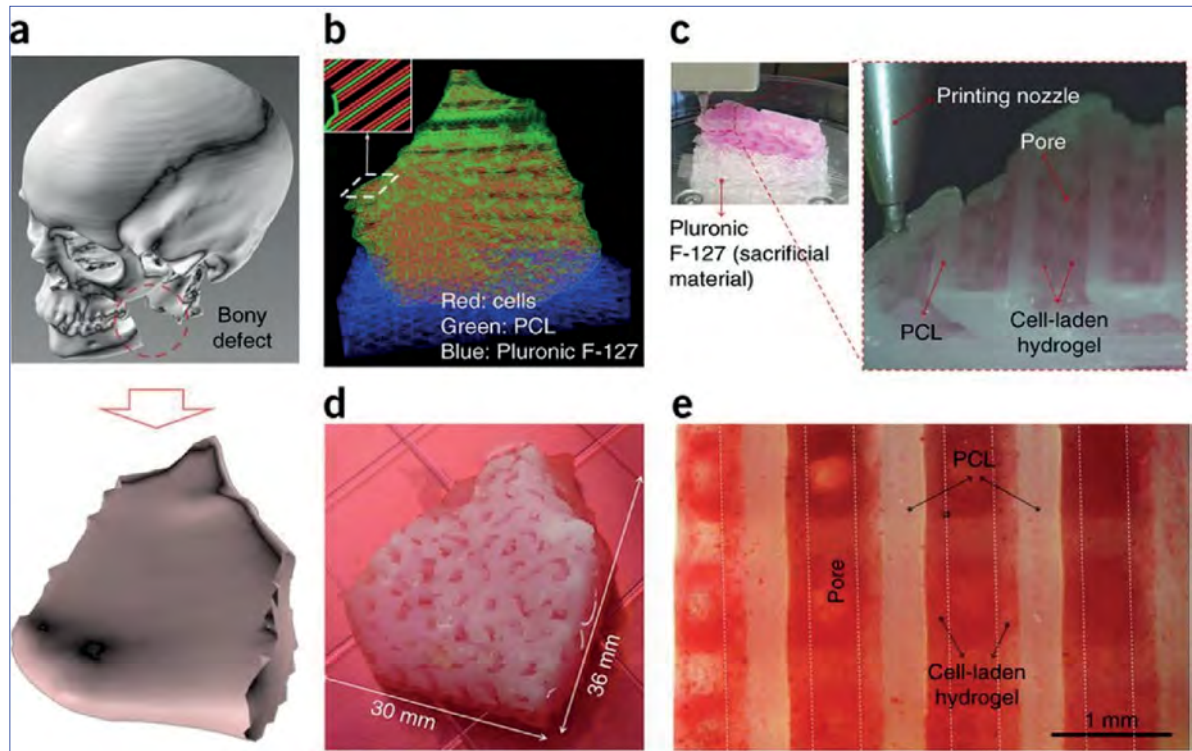
### 6.2. Choice of cells

Many researchers favour the direct printing of cell-laden bioinks, as it allows for niche formation and a level of interaction with the scaffold that cannot be matched when colonizing the scaffold from the outside. The cells that would then be embedded in the bioink can either be functional primary cells with supporting cells (osteoblasts, osteoclasts and perhaps osteocytes) for bone tissue, or stem cells (adipose derived or bone marrow derived mesenchymal). Stem cells require stimulation towards differentiation but contain much more regenerative capacities and are clearly the preference in 3D bioprinting. Survival of the construct when implanted however, would unlikely succeed if there were no additional colonisation of cells from the outside of the construct.

### 6.3. Pore size

To allow such cells to enter and continuously recolonize the 3D bioprinted construct and to allow them to proliferate and function, the construct needs to offer appropriate passages-spaces. This is also necessary to allow nutrients to reach the cells within the construct by diffusion. Keeping in mind the  $\mu\text{m}$  size of pre-osteoblasts, it was established that the appropriate pore size for a vascularized bone matrix would be 200-300  $\mu\text{m}$  in diameter [26]. A larger pore size seems to be met with more vascular differentiation of stem cells whilst a smaller one seems to favour osteogenic differentiation [27]. The established porosity percentage of 90% for bone tissue is left redundant as many researchers have achieved favourable results with





**Figure 11.** Mandible bone reconstruction [36].  
 (a) 3D CAD model recognized a mandible bony defect from human CT image data. (b) Visualized motion program was generated to construct a 3D architecture of the mandible bone defect using CAM software developed by our laboratory. Lines of green, blue and red colors indicate the dispensing paths of PCL, Pluronic F-127 and cell-laden hydrogel, respectively. (c) 3D printing process using the integrated organ printing system. The image shows patterning of a layer of the construct. (d) Photograph of the 3D printed mandible bone defect construct, which was cultured in osteogenic medium for 28 d. (e) Osteogenic differentiation of hAFSCs in the printed construct was confirmed by Alizarin Red S staining, indicating calcium deposition.  
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smaller percentages [4,26]. Also, there is an increased interest in creating a pore size gradient throughout constructs, as this could allow to control growth and mineralisation rates and would fit in the strategy of making scaffold give time-dependant instructions to the stem cells (Time, often referred to as the “4<sup>th</sup> dimension in 3D printing”).

#### 6.4. Cell adhesion

Porosity of the construct also contributes to cell adhesion, albeit more on a micro-level (pores in the scaffold surface = micro-roughness of the scaffold). To this idea, scaffolds have been conjugated with porogens like F-127 [28], NanoHA and  $\text{NH}_4\text{HCO}_3 + \text{Mg}$  [29], often slightly compromising the strength, but improving cell adhesion. Polarity and surface tension of the construct also play a pivotal role in cell adhesion as they determine the hydrophilicity of the construct, which accounts for cell adhesion throughout protein binding to the scaffold. Strategies of reducing the often very negative surface tension (expressed as “surface zeta” value; ex. PLA = -40 mV) include coating the surface with dopamine [28], PEG or Bioglass [30], effectively reducing contact-angles of PLA from  $131.2^\circ$  to  $51.9^\circ$ , making cell-adhesion much easier.

#### 6.5. Ability of the scaffold to send biological cues/ interact with stem cells

Various researchers have experimented with methods of mimicking the interaction between cells and their micro-environment.

Examples include slowing down degradation of bio-ceramics (like Wollastonite;  $\text{CaMgSiO}_3$ ) to provide a steady release of ions, which serves as a bio-cue for bone forming cells [31], adding protein residues such as a cyclic arg-gly-asp chain to bio-gels to stimulate osteogenic differentiation [32], as mentioned above, or even adding plasmid DNA complexes to PLLA/collagen scaffolds to stimulate BMP-2 expression [33].

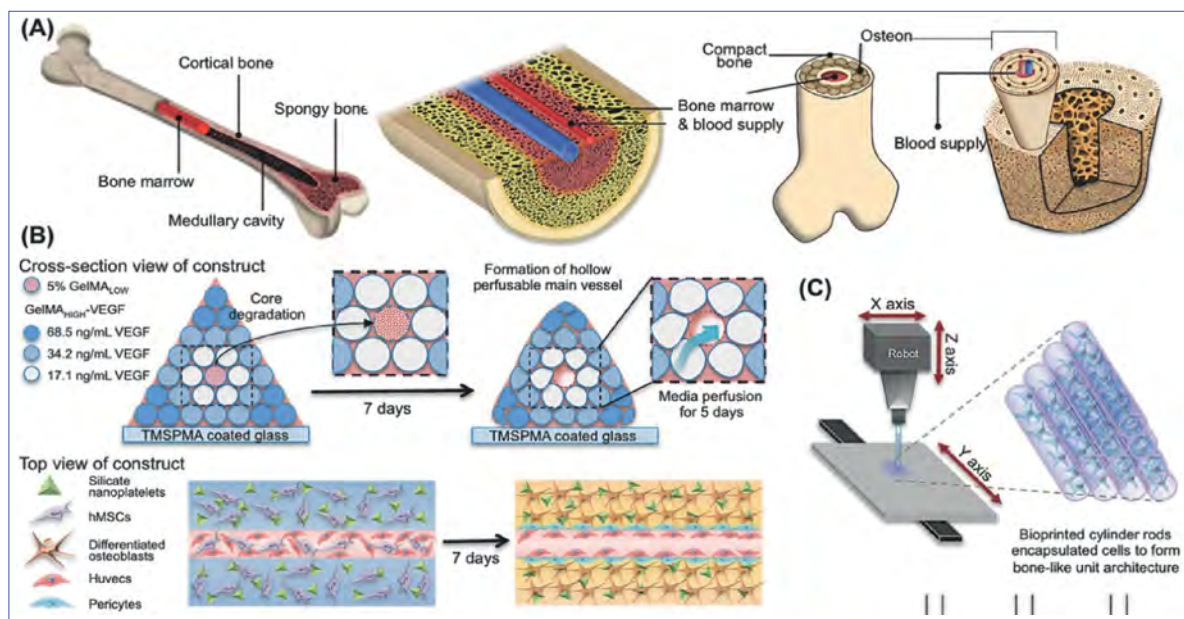
#### 6.6. Ability to develop vasculature

As mentioned above, tissue-engineered bone tissue could not succeed in viability without proper supply of nutrients and oxygen throughout the construct, hence the need for pores. When dealing with a larger bone construct, it would be hard to imagine adequate supply without development of vasculature in the construct.

Actually, this need for vasculature within the engineered bone tissue is presumed indispensable and this is reflected by the considerable amount of researchers simultaneously evaluating bone- and vasculature-formation in their 3D printed scaffolds [4]. Most research truly focussing on (micro)vessel development within 3D bio-printed bone have either (architecturally) created spaces for endothelial cells to arrange into tubes or have aimed to attract (micro)vessel-infiltration from the supporting outside environment.

Examples include silicate bio-ceramics printed into hollow tubes releasing angiogenesis-inducing ions like Mg, Ca, and Si [34], porous CaP scaffolds releasing





**Figure 12.** Fabrication of bone mimetic 3D architecture containing osteogenic and vasculogenic niches [37]. (a) Schematic illustration of complex bone tissue structure. (b) Illustration of the bioprinting strategy for fabricating complex bone tissue architecture. A perfusable vascular lumen lined with HUVECs can be fabricated within a pyramidal bioprinted construct by arranging individual rods of VEGF-functionalized GelMA bioinks with different mechanical strengths. The hMSCs-laden three outer layers of cylinders were loaded with silicate nanoparticles to induce osteogenic differentiation of hMSCs into bone tissue. The VEGF was covalently conjugated into the three outer layers of the cylindrical hydrogels. The concentrations of conjugated VEGF were determined with ELISA as 17.1, 34.2, and 68.5 ng mL<sup>-1</sup>. (c) Scheme of the 3D printing procedure of independent cell-laden cylinders using an automatized and computer-controlled bioprinter. Reprinted with permission by Wiley.

angiogenic growth factors, up to scaffolds with a tube alignment that creates interconnected channels with vascular-like flow patterns [35]. We would like to conclude this design-section with 2 examples of the latest wave of 3D bioprinting research, making use of various, combined techniques to unite the characteristics described above and bundling it all into a direct 3D bioprinted result. The ITOP (integrated tissue-organ printer) by Kang et al. (Figs. 10 and 11), made use of several different cartridges, simultaneously printing combinations of PCL, cell-laden hydrogels of various compositions and a pluronic F-127 component (to stabilize the printing process) into “vascularized cellular constructs of clinically relevant size, shape and structural integrity”. Amongst their productions was a mandible tissue construct (Fig. 11) consisting of PCL/TCP, F-127 and a human amniotic fluid derived stem cell (hAFSC)-laden hydrogel consisting of 35 mg/mL of gelatine (to allow liquification above 37°C), 20 mg/mL fibrinogen (for stability, cell conductivity and cell proliferation) and 10% glycerol (to prevent nozzle clogging). After printing, the fibrinogen was directly crosslinked with thrombin for stabilization after which the rest of the hydrogel components (except for the stem cells of course) were washed away. The outline of the mandible construct was CT image- and CAD model-based and the inner architecture consisted of tubes of PCL/TCP (of 130 μm in diameter) and tubes of hAFSC-laden hydrogel, creating micro-channels of 500 x 300 μm<sup>2</sup>. Cell viability throughout printing (1 day after printing) was shown to be 91 ± 2%, proving this complex printing process did not adversely influence cell viability and after 28 days in culture. Osteogenic differentiation was proven by Alizarin Red S staining for calcium deposition [36]. The need for substantial vasculature within a large

3D bio-printed bone construct was creatively met by Batzaya et al. in their 2017 publication (Fig. 12) in which they presented their pyramidal construct of 28 bio-printed tubes with varying compositions of stem cells, gelatine-methacryloyl, VEGF and Si-nanoparticles. A commercially available 3D bio-printer was used to lay down a central tube of HUVEC- and hMSC-laden gelatine with low methacryloyl substitution (gelMaLOW), which would later gradually degrade to a central open channel, surrounded by 3 layers of 3D bio-printed tubes of hMSC-laden gelatine with high methacryloyl substitution (gelMaHIGH) and a gradient of covalently bound VEGF and Si-nanoparticles. After 7 days in culture, the central tube had become a perfusable lumen with an inner surface of HUVEC’s and an outer surface of supporting hMSC’s, differentiated into supporting smooth muscle cells. The construct was then perfused with an osteogenic medium for 5 days, which supported proliferation and osteogenic differentiation of hMSC’s in the outer tubes, which, 21 days after printing, showed formation of mature bone niches, supported by micro-vasculature [37].

### 7. Conclusion

The sample of recent studies listed above gives us some idea of what is currently being investigated in 3D bioprinting research focussed on bone tissue engineering. It seems clear that obtaining viable 3D printed bone constructs will require a combination of techniques and bio-materials with different characteristics. Clinical application in the field of maxillofacial surgery might not seem up for discussion yet, but research seems to be going the right way at a rapid pace. It is

also remarkable how many of these cutting-edge bone tissue engineering projects are already projecting their experiments towards the maxillofacial area. Amongst bioengineers it seems understood that maxillofacial surgery is a field with great interest in new tissue engineering applications. Several authors describe further elaboration of constructs with closer reproduction of the bone forming niche, more bio-interaction and a higher overall strength of the construct as the major obstacles to overcome in the further development of 3D bioprinting based bone tissue engineering. The further transit toward implantation would also be preceded by more in vitro maturation studies, animal studies and perhaps ethical and regulatory discussions. Aiming at overcoming the patient-inflicted burdens associated with autogenic transplantation, the clinician would be remis not to follow these developments carefully and critically, offering feedback on available materials and constructs and perhaps suggestions in the design process.

#### Author Contribution

SC: provided data gathering, analysis and interpretation. DL: provided data gathering. RJ: provided the core concept, protocol, and revision. CP: provided critical revision and guidance.

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**Questions**

**1. “Inkjet bioprinting” is:**

- a. The first developed type of bioprinting;
- b. The most precise type of bioprinting (highest resolution);
- c. A bioprinting technique that generates droplets of bio-ink;
- d. A jet-laser-based bioprinting technique.

**2. Inducing bio-ceramics printed into hollow tubes releasing ions like Mg, Ca, and Si was found to:**

- a. Increase the strength of the porous CaP scaffolds;
- b. Increasing the E-Modulus of porous CaP scaffolds;
- c. Stimulate osteogenic differentiation of hMSC's;
- d. Create interconnected channels with vascular-like flow patterns in porous CaP scaffolds.

**3. The first 3D printer was invented by:**

- a. Charles Hull;
- b. Jules Pouckens;
- c. Huan Wook Kang;
- d. Thomas Lambrecht.

**4. An appropriate pore size for vascularized bone matrix was established at:**

- a. 20-30 µm, which is within reach of modern 3D bioprinters;
- b. 20-30 µm, which is NOT within reach of modern 3D bioprinters;
- c. 200-300 µm, which is within reach of modern 3D bioprinters;
- d. 200-300 µm, which is NOT within reach of modern 3D bioprinters.