Current trends in 3D bioprinting and endeavours to whole tissue printing.

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Laymen's summary

Bioprinting is a revolutionary 3D manufacturing method which uses a 3D printer to print biomaterials and/or cell containing inks. Bioprinting has become increasingly popular in the field of tissue engineering, as it enables creation of complicated- and small scale models that would not have been possible with traditional methods. With the high demand for tissue- and organ transplants globally, it would be of great value to have a laboratory based method for tissue reconstruction. To date, however, no bioprinted tissues have been approved for transplantation into man. To explain what is holding bioprinting back from being used for patients, we will first provide an overview of available bioprinting techniques and their limitations. With bioprinting the ink has to be 'printable', in addition, the cells also have to be functional and viable in this ink. Therefore, we will touch upon the matrix requirement of the bioprinted ink. Last, developments in bioprinting of various tissue types will be discussed, accompanied by examples of the current state, and problems to overcome to bring bioprinting tissues closer the clinic.

Abstract

Human tissues and organs are complex structures of cells and extracellular matrix components, arranged in a specific composition to excert its functions. In the field of tissue engineering, 3D bioprinting, a type of additive manufacturing, has shown great potential to produce complex structures due to the high spatiotemporal control. The unmatched resolution in printing cell-laden bioinks allows for creation of models with higher physiological resemblance and functionality. With the high demand for tissue and organ transplants, bioprinting is considered a promising tool to fulfil some of these needs. However, bioprinting is currently not routinely utilized in the clinic to produce transplantable tissues, and several issues have to be overcome to enable this. In this Review, we will first provide an overview of model design and printing techniques to give a better understanding of the printing factors to take into consideration. Subsequently, the matrix requirements for printing, and cell functionality and viability will be addressed. Finally, we will discuss current advances made in tissue bioprinting, showcased by some innovative examples, and address challenges in biomaterials, cell biology, and engineering that have to be overcome to bring bioprinting closer to the clinic.

Introduction

In the last decades, a type of additive manufacturing, known as 3D printing, has generated interest of various industries. Subtractive manufacturing – i.e. manufacturing by carving, cutting, or drilling – was and still is the most widely utilized technique to form complex 3D objects. However, the speed, precision, and the ability of a 3D printer to turn an one-of computer created design (CAD) into an 3D object holds great potential. This potential was fairly early seen by some branches in medicine, where 3D printing was used as a tool to create scaffolds for organ transplants for example.

The first patent for a 3D printer was filed by Charles W. Hull in August 1984. The patent for "Apparatus for production of three-dimensional objects by stereolithography" was granted March 11, 1986. It took about a decade before 3D printing was adopted by other industries, however. The implementation of 3D printers in medicine really took off in the mid 90's when this technique was utilized in the production of drug delivery devices and structures for tissue regeneration. The ability to produce polymer based drug release devices with complex internal shapes, allowing for slow drug release, and the ability to control spatial distribution of the drug within the device was revolutionary[1,2]. The high spatial control of the 3D printer was also hypothesize to be beneficial for production of polymeric scaffolds for tissue regeneration. Here, substrate can be accurately dispositioned and complex shapes with

pores and channels can be created, which aid in cellular organization and establishment of vasculature [3,4].

The use of a 3D printer in a biomedical setting was first named ‘bioprinting’ in the early 2000’s. Bioprinting can be defined as “the use of material transfer processes for patterning and assembling biologically relevant materials-molecules, cells, tissues, and biodegradable biomaterials-with a prescribed organization to accomplish one or more biological functions”[5]. The material deposited by a bioprinter is referred to as bioink. Bioprinting was considered an attractive technology as it allows for spatial positioning of cells and biomaterials, for example in engineering tissues and organs. We are about two decades from the early 2000’s, but why is bioprinting not widely used in tissue engineering yet? To answer this question, we will discuss the advances made in bioprinting and give an overview of how bioprinting is currently applied in tissue engineering. First, we will introduce the aspect of model design in bioprinting, followed by an overview of the different bioprinting techniques and their characteristics. Next, we will discuss the importance of matrix composition and modulation of the matrix to create an ‘native-like’ environment. Subsequently, we will give an overview of the current state of bioprinting various tissues, showcased by several examples. Last, we will discuss what is holding bioprinting back from being put into practise and pinpoint the areas in which advancement is required.

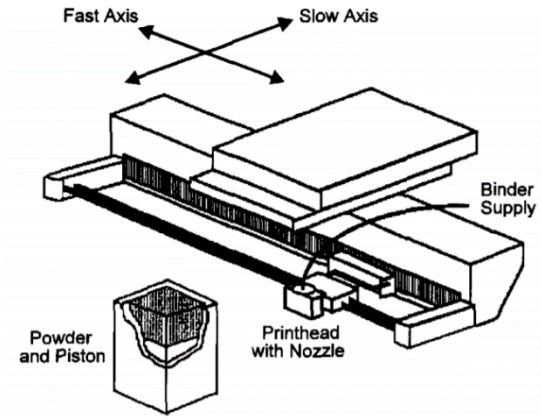

Figure 1. Schematic representation of the inkjet 3D printer used in the mid 90’s[2].

From design to printable output

Computer-aided Design (CAD) is an integral part of bioprinting, as it enables the user to translate their idea into a virtual representation. Subsequently, the actual translation of the CAD design into a product is accomplished by computer-aided manufacturing (CAM). Besides a user created model, there is also the option to acquire a design through medical imaging techniques. Medical imaging techniques such as computed tomography, magnetic resonance imaging, and ultrasound can be used to capture the internal organization and porosity of organs and tissues. By printing a medical imaged CAD design a more realistic model can be created, with the potential to even create patient specific models[6]. The most significant challenge in converting an image to a model, is the reduction of the noise in the image. In imaging, noise refers to random variation in pixel intensity, creating pixels that in reality are not there. For example, speckle noise, the most common type of noise in medical imaging, arises due to environmental factors on the imaging sensor during image acquisition. Since noise reduces the quality of an image, it is necessary to filter out the noise[7]. The challenge here is to remove noise pixels, while retaining the true pixels. When an image is successfully processed, these 2D images can be turned into 3D models by segmenting the regions of interest. With image segmentation, the pixels in an image get divided into regions of interest (ROI), resulting in labelling of objects and boundaries within the image[8]. By compiling all this data, a 3D object can be simulated. After creating a CAD design, this model has to be separated into 2D sections since the object will be printed layer-by-layer. The process of separating the 3D object into 2D sections is termed slicing. With slicing an interpretable code for the 3D printer is generated, consisting of a series of commands instructing the printer the spatiotemporal location to print. For best results, the algorithm created with slicing must also contain information about the properties of the materials used,

as these properties influence how the material behaves when deposited. Whereas these properties are fairly established in commercial 3D printing, these properties of biomaterials are not as established.

In general, software development is not evolving at the same speed as the bioprinting technologies itself do. However, several companies in the bioprinting space are nowadays putting effort into the development of 3D printer operational software. One of the first companies to bring a bioprinter onto the market, Organovo, developed a software package for designing 3D constructs including parameters such as cell type, biomaterial, printing speed and spatial coordination. CELLINK took a slightly different approach, in which the user can simply adjust the speed and flow by which their model will be printed. In addition, their software has a preview function which allows the user to assess the slicing and address potential problems before printing. RegenHU aimed to provide the user with a complete software package to assist in all steps throughout the bioprinting process. Their BIOCAD application provides all the tools needed to create a 3D model, which subsequently can be translated into a printable code with their BIOCAM application. Last, RegenHU's BIOCUT application connects medical imaging with bioprinting, as it analyses medical images and turns the input into a printable code[9].

3D bioprinting techniques

Since the introduction of the stereolithography 3D printer, several other printing techniques have been developed. However, not all these techniques are applied in a biomedical setting. Factors such as the cell type being used, optimal ink for these cells, printing characteristics of the ink, and required precision are not compatible with all printing techniques. Still, many things can be learned from these printing techniques and future endeavours with these techniques cannot be excluded as the field of bioprinting evolves in an unprecedented pace.

Fused deposition modelling (FDM) is one of the most popular 3D printing techniques, its utilization in bioprinting is however very limited. In FDM, a thermoplastic polymer is heated to a molten state, extruded through the nozzle of the printer, and deposited in a layer-by-layer fashion onto a platform. The x, y, and z coordination of the nozzle is computationally scripted and controls the printing of a 3D shape[10]. The spatiotemporal control of the nozzle in the x, y, and z dimension is one of the main features of FDM. This control would be highly advantageous in bioprinting, as with this controllability high control over porosity of constructs and channel sizes within the construct can be created (e.g. vasculature)[11]. Limiting factors of FDM in bioprinting are the limited thermoplastic polymer selection and the high temperatures required to melt these thermoplastic filaments for extrusion[12]. Furthermore, FDM with current biocomposite filaments is not conform the required mechanical strength, spatial accuracy, and layer adhesion[13]. However, progress is being made as new materials and combinations with biocompatibles (mainly for scaffolds) are tested and the number of new FDM filaments is continuously increasing[11].

In addition to FDM, selective laser sintering (SLS), selective laser melting (SLM), and electronic beam melting (EBM) have not taken off in bioprinting. These powder bed fusion techniques use intense laser or electron beam energy to fuse powder particles together by heating the particles to above the glass melting point. Layers can be built by lowering the powder bed platform, applying new powder and repeating the process. These methods display high resolution, as resolution of these techniques is dependent on laser beam diameter, focusability (dependent on the wavelength), powder particle size, and the increments by which the laser can be moved[14]. These techniques are typically used to generate mechanically strong objects. Most techniques that create mechanically strong constructs, typically require high temperatures or toxic solvents to facilitate printing[12].

Therefore, these techniques are not suitable for printing cells since viability would be greatly affected. However, these techniques can be utilized to create scaffolds to seed cells on.

The first of 3D printing techniques, stereolithography (SLA), is only sparingly applied in bioprinting. With SLA, an UV or electron beam photocrosslinks a liquid resin onto a platform (Figure 2). This platform can be moved along the z-axis, which allows liquid resin to flow on top of the first layer, enabling crosslinking of another layer on top. Commercial SLA printers are designed for printing hard plastics, with post-printing treatments such as heating or photocuring in an UV oven to enhance mechanical strength. For biomaterials, the aforementioned treatments are all cytotoxic to some degree, making this technique less suitable for bioprinting[15]. The main limitation of SLA in bioprinting is that this technique is not setup to accommodate soft biomaterials. The soft- and hardware used in this technique need various adaptions to be compatible with bioprinting[16]. The bioprinting community would greatly benefit from a SLA compatible method, as SLA can with high precision illuminate the liquid bioink and does not require physical contact with the ink. The latter would be extremely valuable, as shear stress on cells is one of the major limitations of the extrusion based techniques which are the current bioprinting standard.

Recently, B. Grigoryan *et al*. adapted the traditional SLA technique to enable 3D printing of biological materials and even added a multi-material element[16]. With their multi-material stereolithography (MMSLA) printer this group aimed to build a printer that allows for printing of different cell types in different bioinks. The inability to print different cell types and bioinks simultaneously in traditional SLA bioprinting was one of the elements holding SLA back from being further implemented. With this multi-material approach a more *in vivo* representative model can be generated, with correct spatial distribution of the different cell types. In addition, printing of seam- and voidless structures, often a problem in extrusion based bioprinting (EBB), was hypothesized to greatly benefit cell migration between domains.

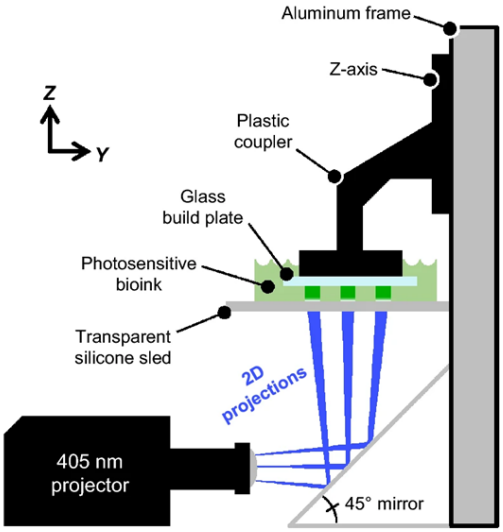

Figure 2. Schematic representation of the stereolithography bioprinter developed by Grigoryan et al. CC BY 4.0

The EBB technique pressure assisted microsyringe (PAM) has been the main bio-based printing technique over the years. With this technique, a bioink is loaded into a syringe and subsequently dispensed onto a building platform by either piston, pneumatic, or screw driven force. Several layers of cell-laden bioink can be dispensed on top of each other to create an advanced predefined shape. The rheological properties of a bioink, i.e. flow properties, are one of the most important factors in bioink selection. The rheological properties are determined by the combination of the polymer, excipients, and the formulation of the aforementioned ingredients. With good rheological properties, high control over the extruded ink can be achieved, resulting in printing of models with optimal resolution. Another important factor on rheology of the bioink is the shear thinning property (i.e. decrease in fluid viscosity with increase in shear stress). With EBB, bioinks are exposed to high pressures at the nozzle, as the ink is forced through a small diameter orifice. Viscosity of the ink should not be too high, resulting in clogging of the nozzle, and not be as low to the point the ink is deposited uncontrollably. Sadly, there is not a way to measure the viscosity of the polymer during extrusion and therefore the acceptable viscosity range is unknown. A more detailed

approach to estimate filament viscosity is well reviewed by Azad *et al*[17], which can be used to estimate print quality of a selected ink. The ease of use is the main advantage of EBB. These bioprinters are relatively cheap, have a wide range of available polymers, and can print at room temperature. In addition, EBB can print hydrogels with high viscosity, beneficial for printing of high cell densities (Table 1). The latter is an important factor in tissue transplantation, where implants require high, *in vivo* like, cell densities. Drawbacks related to this technique are the low cell viability due to shear stress in the nozzle, prone to clogging, and low resolution as the resolution is for a great extend determined by the nozzle size (the extruded filament is 150 – 300 µm in diameter)[18].

Table 1. Summary of the parameters of the most common bioprinting techniques [18,19]

	Extrusion	**Inkjet**	**Laser-assisted**	**Stereolithography**
Bioink viscosity (mPa/s)	30 – 6E7	3.5 – 12	1 – 300	No limitation
Cell density (cells/ml)	High	< 10E6	< 10E8	< 10E8
Cell viability (%)	40 – 80	> 85	> 95	> 85
Print speed	Low	High	Medium	High
Resolution (µm)	200 – 1000	10 – 300	10 – 100	High
Cost of printer	Medium	Low	High	Low

Another common bioprinting approach is inkjet. With inkjet bioprinting, cell-laden ink is deposited droplet-by-droplet onto a platform. Droplets can be printed in a layer-by-layer fashion, however, rapid crosslinking between layers is required since low viscosity bioinks are used in inkjet printing. The printer deposits droplets by either thermal or piezoelectric force. With thermal force, the temperature of the heating element is increased to approximately 300 °C, resulting in partial vaporization and formation of a small bubble in the ink filled chamber. The bubble expands and subsequently bursts, resulting in a pressure pulse great enough for the bioink to overcome the surface tension to be ejected out of the nozzle. With piezoelectric force, the piezoelectric actuator induces deformation of small crystals in the bioink, increasing the volume in the bioink chamber, resulting in a pressure pulse induced ejection of bioink out of the nozzle. Because the induced changes in chamber volume are small, the created droplets are only 50-75 µm in size. This results in printing with medium resolution, high cell viability and good printing speed. Because of these beneficial characteristics, efforts have been put into printing multiple materials with inkjet, named Polyjet. Polyjet is designed to simultaneously jet and photocure the ink. These features add to the utility of this technique, making fabrication of more complex models with multiple materials/cells possible[20]. However, the main limitation of inkjet remains, which is a low viscosity bioink requirement, only allowing for printing of low cell densities.

Similarly to inkjet bioprinting, laser assisted bioprinting (LAB) is a droplet based printing technique. Here, a laser is projected onto an absorbing layer composed of a metal film, inducing vaporization in the underlying bioink layer. Vaporization will create a pocket in the bioink layer, upon which collapse the bioink droplet will transfer onto a substrate layer. This nozzle-free approach significantly reduces stress on the cells, resulting in high cell viability. Bioinks with high viscosity and high cell density can be printed, without compromising cell viability[21]. As with other laser based techniques, small droplets can be created which allow for printing with high resolution. Despite the advantageous properties of LAB, the high price, complicated operating procedures, and many materials required hold back this technique from being widely utilized.

Matrix requirements for printing cellularized constructs

When printing living cells, the goal is to provide the cells with a matrix simulating their native environment. When this matrix contains biomaterials, cues, factor, or living cells, this ink is referred to as a 'bioink'. The backbone of a bioink is either a synthetic- or biopolymer. In bioprinting, most applications make use of a (multi) biopolymeric backbone, as these polymers are more biorelevant. Most polymers used in bioprinting are hydrophilic, creating hydrogels with the ability to retain large quantities of water. Hydrogels have relatively low structural rigidity because of the low polymer concentration[22]. With this lower polymer concentration higher porosity is established making these hydrogels highly permeable to water-soluble compounds, nutrients and oxygen, creating an ideal environment for cell culture. However, these mechanical weak hydrogels reduce printability of complex 3D structures. To successfully create a 3D structure, the right physical, mechanical, and rheological properties of a bioink are essential, facilitating layer-by-layer printing. This is where synthetic polymers can provide a solution, as primary backbone of the hydrogel or as a scaffold to load the hydrogel into. Polyethylene glycol (PEG) is one of the most widely used synthetic polymer backbones. PEG can link various functional groups, it has good mechanical tunability, shows good safety, biocompatibility, and hydrophilic propperties[23]. The latter is what favours PEG over other synthetic polymers such as polycaprolactone (PCL) and polylactic-co-glycolic acid (PLGA) which are hydrophobic[24]. PCL and PLGA are often used for scaffold approaches, where cell-laden hydrogels can be loaded into.

In bioprinting, the main biopolymers utilized are gelatin, collagen, fibrin, and alginate. From these biopolymers, alginate is least bio-relevant, as alginate is extracted from sea weed. However, alginate allows for mild crosslinking conditions and rapid gelation, which is beneficial to cell viability. Furthermore, alginate lacks cell-interactive domains, making it an interesting option when a non-adhesive environment is desired[25]. The choice between gelatin, collagen, or fibrin will be based on the cell type used. Collagen and fibrin are extracellular matrix (ECM) proteins which are used in gels where a biomimicking environment is desired. ECM proteins such as collagen, fibronectin, and laminin mediate in proliferation and differentiation by binding to transmembrane integrin receptors[26]. Collagen is one of the main components of fibrous tissues (e.g. tendons, ligaments, and skin) and is the most abundant protein in the human body. In a 3D structure, collagen can aid in cell attachment and migration, generating a spatial distribution of cells which mimics the *in vivo* environment[27]. A major limitation of collagen as a backbone in bioprinting is the slow gelation rate. This slow gelation rate accompanies several issues, such as troubled layer-by-layer disposition and homogeneous distribution of cells within the printed construct (sedimentation of cells in gelated state). Gelatin, which is a collagen derivative, has a similar molecular composition to collagen and therefore fulfils similar functions as collagen *in vitro*. In addition, gelatin is cheaper and has higher solubility while maintaining its functionality for ECM interactions[27]. Fibrin structures are realised by crosslinking of fibrinogen with thrombin, forming a fibrous 3D network[28]. *In vivo* this interaction takes place during wound healing, creating a network of filaments which form a soft complex that allows for a high degree of deformation. In addition, fibrin promotes cell growth and proliferation which contributes to wound healing. Dependent on the concentrations of fibrin and fibrinogen, fibrin can be 500 to 1000 times softer than collagen, making it an excellent choice for soft tissue[28].

However, these biopolymers have in common that they are rarely printed in their native form because of their poor mechanical properties. As an example, gelatin physically crosslinks at room temperature, it however solubilizes when exposed to body temperature (37 °C)[29]. Mechanical stiffness is known to play an important role in cellular behaviour and differs between native tissues. Therefore, several strategies have been employed to improve

mechanical properties of constructs with a biopolymer backbone. First, multiple biopolymers can be co-printed to enhance printability or post-printing structural stability. For example co-extrusion of fibrin and gelatin: fibrin is non-shear thinning (i.e. the mixture does not become less viscous when pressure is applied) and is therefore not printable, however, gelatin has favourable gelation properties making co-extrusion possible[28]. Second, additional ECM components can be added to tune construct stiffness. To exemplify, fibrin construct stiffness can be modulated by printing a blend of fibrin and hyaluronic acid (HA). The addition of crosslinkable HA chains can increases overall stiffness of the construct. In addition, HA is present in most connective tissue and is present in the ECM of cartilage tissue, making it a preferred choice in models where wound healing, nerve regeneration, and neuronal and glial development is required.

Crosslinking to modulate mechanical properties

By the addition of a crosslinking component to the biopolymer hydrogels, the stiffness of the construct can be tuned. Different tissues have different Young's modulus, which is a geometry-independent measure of tissue stiffness. Functional hydrogels stiffness between 0.1 – 1 kPa, 30 – 50 kPa, and >500 kPa have been shown for brain, tendon, and cartilage respectively[30]. In order to crosslink biopolymers, chemical modifications are needed to introduce linkages. The most common conjugation reactions here are amidation, esterification, etherification, and carbamate formation. By subjecting amino acids containing hydroxyl, carboxyl, or amines to these conjugations, various types of chemical groups can be introduced to the biopolymer[31]. Subsequently, these introduced chemical groups can be crosslinked together by various approaches such as: photocrosslinking, chemical crosslinking (covalent bonding), physical crosslinking (non-covalent bonds), enzymatic crosslinking, and thermal crosslinking. Photocrosslinking is probably the most popular crosslinking technique, which is due to its ease to use and spatiotemporal control. This technique makes use of photoinitiators in the bioink. Upon irradiation, these photoinitiators get dissociated, leading to formation of free radicals, which react with vinyl groups of the polymer and binds the polymers[31]. The degree of crosslinking depends on factors such as biopolymer concentration, light intensity, exposure time, and photoinitiator concentration[32].

A popular approach in photocrosslinking is methacrylation of biopolymers prior to crosslinking. Methacrylic anhydride (MA) reacts with free amino groups (mainly amines) on biopolymers such as gelatin (GelMA) and HA (HAMA), resulting in methacrylamide and methacrylate sidegroups[33–35]. These groups, named methacryloyl groups, can be crosslinked together through photopolymerization[36]. The mechanical strength of these hydrogels is tuneable, as the amount of added MA is proportional to the degree of crosslinking. On the contrary, a lower degree of methacrylation (i.e. ratio between the number of methacrylate groups and number of unreacted amine groups) allows for greater swelling of the construct and therefore larger pore size, which is a favourable cell environment. This is however at cost of printability. As touched upon here, there are many factors in photocrosslinking that have to be taken into consideration to create an environment most suitable for the cells being used. More information can be found in a very extensive review by S. Knowlton *et al.*

Physical crosslinking utilizes ionic interactions to form crosslinks between polymer backbones. Ionic crosslinking can be initiated by either metal ions or electrostatic. With the addition of a metallic cation (i.e. a positively charged ion), two carboxylic groups of adjacent polymers can be bound. The most popular approach here is to form a ionically crosslinked hydrogel by the addition of $CaCl_2$, with calcium being the most stable cationic agent[37] and the $CaCl_2$ combination resulting in high solubility in water. Because this type of crosslinking utilizes ionic interactions between carboxylic groups, only polymers containing carboxylic groups can be crosslinked by this technique. In addition, this type of crosslinking results in

relatively weak mechanical properties and the release of metal ions can cause toxicity to encapsulated cells. Benefits are the mild and rapid crosslinking that can be applied at room temperature and physiological pH. In addition, there is noncovalent binding through hydrogen bonds, hydrophobic interactions, dipole-dipole interactions, and more recently DNA hybridization. Because noncovalent bonds are relatively weak, this approach is most suitable for applications where a very soft structure is required such as brain or lung[31].

Compared to the crosslinking techniques above, the use of thermal and chemical crosslinking is limited. Thermal crosslinking of polymers can be accomplished by exposing a polymer solution to either heating or cooling. The mechanism of crosslinking differs per polymer, for example: by decreasing the temperature of an agarose solution from >40 °C to ~32 °C the agarose coil conformation changes to a helical structure, which link together. Similarly, GelMA transitions from liquid at 37 °C to a gel at <21 °C, due to a coil to helical transition[38]. The simplicity of thermal crosslinking makes this technique an attractive approach, the slow gelation time, varying degree of crosslinking, and crosslinking temperatures can limit its utilization, however. Contrary to the other techniques, chemical crosslinking directly modifies the biopolymer to enhance hydrogel formation. Chemical crosslinking entails covalent binding of polymer chains, which can be done through reactions such as Schiff base, azide-aldehyde coupling, azide-alkyne cycloaddition, acylhydrazone, and diels-alder linkage[32].

Bioprinting of soft tissues

Now we have established the various printing techniques, matrixes, and crosslinking techniques, we will highlight some of the latest achievements made in bioprinting mediated tissue engineering. In tissue engineering, bioprinting mostly focusses on development of skin, blood vessel, cardiac, and bone and cartilage models.

Skin

In the field of tissue engineering, most bioprinting related research has probably been in developing skin constructs. Bioprinted skin can function either as skin grafts or as a dressing to aid in wound recovery. Human skin consists of four layers with different cell types in each layer. The outer layer of the skin (epidermis) is composed of mainly keratinocytes, with the top of this layer consisting of more mature cells, while the bottom is comprised of new, proliferative, cells. The second skin layer (dermis) is composed of three major cell types – fibroblasts, macrophages, and adipocytes. The ratio of collagen I or III in the ECM of the upper- or lower part of the dermis determines the elasticity and mechanical strength of these parts of the skin. In addition to these components, skin also contains blood vessels, sweat glands, nerves, and hair follicles, all with intricate spatial arrangements which make printing of such a construct rather challenging[39].

Hafezi *et al*. used an EBB approach to print a human fibroblast and keratinocyte loaded chitosan (CH)-PEG bioink[40]. Here, a first supporting layer of alginate was printed and crosslinked by $CaCl_2$. Next, two subsequent layers of genipin crosslinked CH-PEG bioink loaded with fibroblasts and keratinocytes were printed. Crosslinking with genipin, a naturally derived agent that links free amine groups of the biopolymer CH, was done to obtain good viscoelasticity properties. High keratinocyte viability was reported (>85%). However, this is compared to general cell viability after EBB printing (40 – 80%) and was only measured for 7 days. Furthermore, it was reported this bioink did not support proliferation of cells, which decreases the utility of this ink combination drastically. If this ink does not support proliferation, it can only be used as a short term solution such as a dressing, as for a more prominent solutions cells have to be able to renew.

For *in vitro* research applications, Liu *et al*. used an EBB approach to print a fibrin based hydrogel loaded with fibroblasts, induced pluripotent stem cell (iPSC) endothelial cells, and pericytes (cells embedded in the walls of capillaries, formed by endothelial cells) [41]. The approach with a fibrin based hydrogel shows matrix requirements were taken into consideration, as fibrin based hydrogels have shown to prevent tissue contraction, which is seen in collagen based hydrogels. The ink was printed in a transwell with a biodegradable PLGA scaffold which served as a vasculature. The construct was crosslinked by addition of thrombin and after 7 days of dermis formation the epidermis, consisting out of keratinocytes, was seeded on top of the construct. A fully differentiated and stratified epidermal layer was confirmed by marker expression. Angiogenesis in the dermis became evident after 17 days of culture, indicative of vasculature formation. The work here shows good progression in skin bioprinting, with formation of a mature epidermis and vasculature, however the full range of *in vivo* skin components in not captured.

A more sophisticated approach to print an *in vitro* vascularized skin model was taken by Kim *et al*[42]. In short, a PCL transwell was printed as a perfusable base, on top of which a PCL mesh with perfusion chamber was printed. Next the hypodermal compartment, consisting out of preadipocytes in a fibrinogen hydrogel, was extruded on top. Subsequently, human umbilical vein endothelial cells (HUVECs) and thrombin, loaded in a 10% gelatin hydrogel, were printed on top of the hypodermal layer in a cylindrical structure to form a vasculature. This approach is rather innovative – the gelatin will maintain its shape during fabrication of the construct, but will liquidify when cultured in 37 °C, leaving a hollow channel under the subsequent printed layer due to crosslinking by thrombin. This referred to subsequent layer is the dermal compartment, a human dermal fibroblast encapsulated fibrinogen hydrogel. After 7 days, primary human epidermal keratinocytes were printed on top of the construct using an inkjet modulus. The inkjet modulus offered uniform distribution of keratinocytes, as the ink printed has a relative low viscosity. During culture the vasculature channel was connected to the chamber of the PCL transwell, which was equipped with a peristaltic pump that allowed for perfusion of the model. Through this channel several types of medium were supplemented at different stages of skin development. This resulted in successful skin maturation, demonstrated by a stratified epidermal layer, secreted ECM composition in the dermal layer, lipid droplets in the hypodermis, and an endothelium lined vascular channel. Furthermore, their vascularized model showed higher similarity to native skin compared to a normal skin model (composed of dermis and epidermis), determined by the functional markers p63 and keratin 19.

Jorgensen *et al*. made a great effort to recapitulate native skin, as human keratinocytes, fibroblasts, melanocytes, dermal microvascular endothelial cells, follicle dermal papilla cells, and adipocytes were printed in one construct[43]. To elaborate, it was reported that 61% of EBB constructs only use a single cell type in their model[39]. In this model a fibrin based bioink with addition of gelatin, glycerol, hyaluronic acid, and fibrinogen was printed. The cells were resuspended in three different bioinks: Keratinocytes and melanocytes in the Epidermis, fibroblasts; follicle cells; and endothelial cells in the Dermis, and preadipocytes in the hypodermis layer. A biomimetic model was printed by subsequent printing of the hypodermal, dermal, and epidermal layers. Thrombin was added to the hydrogels to crosslink the fibrinogen. After 4 days, grafts were implanted onto full-thickness wounds (wound extending beyond the epidermis and dermis) and after 21 days the wound areas were analysed. Compared to the hydrogel only and wound only control conditions, bioprinted skin was found to accelerate wound closure, with rapid formation of an epidermal barrier. Collagen remodelling in the bioprinted grafts resulted in an ECM containing a balanced ratio of young and mature collagen fibres, as would be found in a recovering wound. Compared to the hydrogel control condition, increase in capillary lumen was found, which should aid in

distribution of nutrients and toxins. At day 21, staining for adipocytes in the bioprinted graft was negative, while a strong response for the mesenchymal marker vimentin was found. It was hypothesized that, when placed into the wound, preadipocytes differentiated to a mesenchymal cell type such as fibroblasts. The effect of his differentiation is hard to estimate, as the dermal composition underlying the graft is unknown. Therefore, it is unknow to what state the native tissue recovered. Last, the added follicle dermal papilla cells did not mature, as no hair follicles were detected on day 21.

All in all, bioprinting is a promising tool for engineering skin tissue. With bioprinting, cells can precisely and uniformly be arranged in a spatial arrangement, something that is not easily achieved by manual processes. However, improvements need to be made to decrease recovery time for printed cells and more attempts have to be made to implement all components of human skin (e.g. glands, follicles, etc.).

Blood vessels

There has been an increasing interest in printing blood vessels, as vascular diseases are amongst the leading causes of mortality worldwide and prevalence is steadily increasing. The standard of care for these patients is using autologous grafts to bypass affected vessels, however, a third of these patients have multivessel disease and therefore lack vasculature suitable for a graft. An ideal bioprinted blood vessel is biocompatible, hemocompatible, and has the correct tear and burst strength. In physiological conditions, blood vessels consist out of smooth muscle cells surrounded by ECM proteins such as collagen and elastin. Subsequently, for regenerative integration, ECM and cell migration must be established, leading to fading of the host-graft interface and establishing homeostasis[44].

Physiological arteriovenous channels consist out of three layers – the adventitia, media, and intima layer – lined with human dermal fibroblasts (HDF), vascular smooth muscle cells (VSMC), and endothelial cells (EC) respectively. Advances made in creating these three layered constructs used either extrusion or droplet based techniques. Xu *et al*. composed a double walled supporting scaffold out of a silicone elastomer to provide sufficient mechanical strength to the vessels[45]. The silicone elastomer (SE1700) has good biocompatibility and has small micropores for nutrient diffusion and cell migration. A four-nozzle 3D printer was used to print VSMCs loaded dECM in the double walled scaffold, followed by perfusion of the inner channel of the scaffold with a HUVEC suspension. Last the construct was immersed in in a HDF suspension, allowing the HDFs to adhere to the outer wall of the scaffold. Imaging after 48 hours revealed maintained structural integrity and high viability of cells. However, the complex and time consuming production process (approximately 36 hours) is a significant drawback.

Similarly, Schöneberg *et al*. used a multi-headed 3D printer, however here an inkjet based approach was taken to print blood vessels (Figure 3) [46]. With this model the authors showed their ability to create a realistic *in vitro* model by using natural materials. Cell viability was acceptable, averaging 83.2% at day 0 and increasing to an average of 91.3% at day 4. A model with adventitia, media, and intima layer was created, with a wall thickness similar to small arteries and veins. Compared to the general standard, a rather ingenious approach was taken to make full use of bioinks with different characteristics. The utilization of two synergetically working print heads, one with crosslinking solution and the other with cell-laden bioink, is still rather new. The subsequent liquification of the inner core and adherence of HUVECs to the medium layer testifies of good knowledge of options to reach the required model composition.

The development of bioprinted blood vessels has progressed greatly in recent years, demonstrated by several models displaying a triple-layered arteriovenous channel. One of

the main challenges is to create a vessel that accomodates changes in vessel diameter to regulate blood flow and pressure. Creating a blood vessel with a wall thickness similar to that of a blood vessel *in vitro* does not mean it has the same mechanical and elastic strength. Furthermore, a transition to relevant cell types has to be made, for example, HUVECs are often used as surrogate EC. Another interesting addition to current models would be flow, as various cell types have shown to benefit from flow to align and find their spatial orientation.

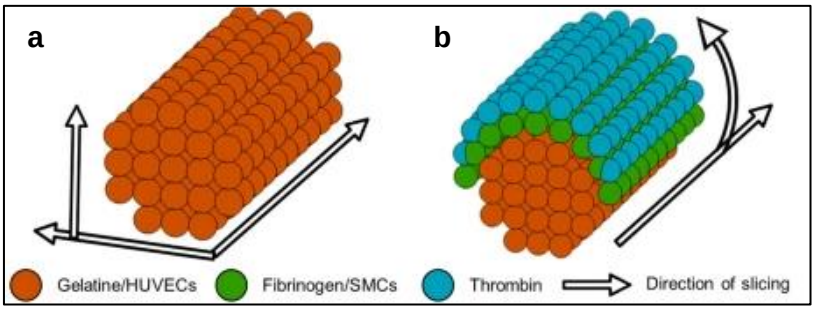

Figure 3. Blood vessel printing approach by Schöneberg et al. a) First, the inner core, consisting of HUVECs suspended in gelatin, was printed into a 5 °C cold reactor to gelate the gelatin. Droplets were printed in a circular shape and stacked on top of each other to create a 3D structure. b) Next, the media layer was printed on top of the inner core and this was done by alternating between two print-heads – first a drop of thrombin crosslinker was printed, followed by a drop of SMC loaded fibrin, resulting in direct crosslinking (Figure 3b). Subsequently, the gelatin is liquified and this suspension is kept in the construct for two to four hours to allow HUVECs to sediment and attach to the surrounding layer. While letting the HUVECs sediment, the surrounding fibroblast layer was cast in a hydrogel mixture of fibrinogen and collagen, crosslinked by addition of thrombin. Last, the sacrificial gelatin core was washed out, creating a three layered vasculature[46] *(CC BY 4.0).*

Cardiac tissue

There is a large demand for cardiac tissue as heart failure remains to be one of the leading causes of death. New modalities such as 3D printing offer a high degree of complexity and can print advanced ECM compositions which is a welcoming feature for cardiac tissue engineering. From the different types of cardiac tissue, most efforts have been put into printing myocardic patches. For myocardic tissue, the mechanical modulus (i.e. Youngs modulus) is essential for cardiac myocytes to develop and mature. In cases where the preferred bioink does not possess the required modulus, additional measures can been taken to assure compaction of cardiac tissue, such as scaffold base approaches[47] or needle arrays[48]. Cardiac tissue engineering has made great progress in recent years, to the point several groups have tested their bioprinted heart patches *in vivo*.

Bejleri *et al*. made use of GelMA as a supporting material to their decellularized cardiac extracellular matrix hydrogel. The addition of crosslinkable GelMA increased the modulus up to 3000 – 5000 Pa, which is within the physiological myocardium range. Neonatal human cardiac progenitor cell-laden constructs were printed in grids of 90° intersecting filaments, and showed high viability and proliferative capacity *in vitro*. In rat, subsequent epicardial attachment of the bioprinted patches could be maintained up to 14 days and integration was indicated by host vascular formation[49]. Similarly, Maiullari *et al*. printed grid patches of PEG-fibrinogen laden with HUVECs and iPSCs derived cardiomyocytes. This group made use of a microfluidic printing head, allowing them to simultaneously extrude multiple bioinks. By simultaneous extrusion of cell-laden bioink and $CaCl_2$, hydrogel fibres were formed immediately upon extrusion. With this approach the cardiomyocytes orientate along the printing direction, which is crucial for cardiac tissue functionality. Without coordinated spatial organization the culture would generate multidirectional contraction, which net force is zero. After 14 days of subcutaneous engraftment, the *in vitro* developed vasculature network showed integration with the host's vasculature and cells were highly viable[50].

Cell-free patches offer a more simplistic approach to induce tissue regeneration. Cell-free patches can be placed to prevent ventricular remodelling caused by myocardial infarction, as ventricular remodelling can reduce cardiac contractility. Biodegradable polymers such as PCL and poly glycerol sebacate (PGS) have shown good biocompatibility, mechanical strength, and elasticity, making them suitable backbones for cardiac patches. The role of the patches is to temporarily sustain the tissue architecture, allowing recovery of the native tissue[51]. As seen in other tissues, implanted scaffolds can be designed to additionally function as drug release devices. An innovative cardiac patch was developed by Feiner *et al*., which enabled recording of the patch's cellular electrical activities, and subsequent stimulation of activities by subsequent release of biofactors[52]. Recently, Huang *et al*. build upon this format and composed a delivery system out of a PCL/PGS scaffold, increasing the strength and elasticity to meet the requirements of cardiac tissue[53].

Both cell-laden patches and cell-free patches have their advantages and disadvantages. Maintenance and engraftment of cell-laden patches has shown to be difficult. However, a cell-laden patch can actively support the beating heart, as CM-laden patches have shown spontaneous contractile force[54]. In addition, cell-laden patches have shown to accelerate the tissue regeneration process. Cell-free patches lack these cell mediated benefits, however, because cell-free patches do not have to accommodate cells a wider range of polymers can be used, which in turn allows for more mechanical tuneability.

Bone and cartilage printing

Cartilage

Engineering and replacement of cartilage tissue has been widely investigated since damaged cartilage does not heal or regenerate[55]. Current restorative options for articular cartilage are marrow stimulation and osteochondral grafting. However, these options are short term solutions, as the native architecture is not resembled. Current cell-based approaches result in disorganized cell structures which have poor durability[56]. A technique with high spatiotemporal control, such as 3D printing, that enables a highly accurate reconstruction of native tissue could provide a solution.

In cartilage bioprinting, alginate and agarose have found to support hyaline cartilage, whereas GelMA and PEGMA support fibrous cartilage tissue better. An important characteristic of connective tissue is its mechanical strength. Whereas soft tissue engineering mainly focusses on providing cells with an optimal hydrophilic matrix, mechanical strength is an additional requirement in connective tissue engineering. Bas *et al*. found a PEG/heparin hydrogel loaded into a 3D printed 200 µm fibrous PCL scaffold to display mechanical properties highly similar to that of articular – a type of hyaline – cartilage. In addition, fibre reinforced constructs showed enhanced chondrocyte viability and differentiation compared to non-reinforced conditions. Similar findings with respect to mechanical strength and chondrocyte functionality were reported by Visser *et al*. Here, 200-1000 µm pore PCL scaffolds were loaded with chondrocyte-laden GelMA, which increased stiffness up to a 50 fold[57]. With a PCL scaffold the time between transplantation and regeneration of native tissue can be bridged. To clarify, a hydrogel within a scaffold will degrade within months, whereas the scaffold will take years to degrade. This allows new tissue to form within the support scaffold and to slowly increase its mechanical strength[57]. By implementing hyaluronic acid methacrylate (HAMA), which is a more physiologically relevant cartilage matrix, Mouser *et al*. optimized bioactivity of such a chondrocyte-laden PCL scaffold[58]. To further develop a biomimetic scaffold, Y. Sun *et al*. developed a scaffold with a pore size gradient, since chondrogenesis is promoted by small pore sizes (100-200 µm) while osteogenesis and angiogenesis are inhibited in small pores[59].

A challenge in many fields of tissue engineering is to produce vascularized constructs of a physiologically relevant size. For this purpose, Kang *et al*. developed a multi-cartridge extrusion based bioprinter to print a combination of cell-laden hydrogels, supporting PCL polymer, and sacrificial Pluronic. This group was able to print full-sized tissues such as an ear, bone fragment, and a 15 x 5 x 1 mm muscle construct. These constructs showed to be viable and to mature *in vivo* during a culture time of 5 weeks. The ear construct, measuring 32 x 16 x 9 mm, showed signs of tissue formation and no signs of necrosis, indicating successful vascularization[60]. The strong focus on cell viability and quantitative analysis limited this study. Implantations were made in ectopic sites, which therefore did not allow for extensive functional assessments. However, the setup of a printer that can print viable human scale tissues is promising.

An *in situ* approach was taken by Di Bella *et al*., developing a cartilage biofabrication technique to repair a cartilage defect in a single-session surgery. Here, chondral defects in knees of male Merino cross sheep were created. Subsequently, each defect was treated by either the 'biopen' or with control methods. The biopen – an extrusion based system with a dual cartridge, dual core nozzle – simultaneously printed the shell and the cell-laden core. A combination of GelMA and HAMA was used to print both the inner core and shell, where the shell was loaded with photoinitiator and the core with mesenchymal stem cells. Compared to a preconstructed scaffold, non-treated control, and microfracture – a procedure in which multiple small incisions are made to stimulate natural healing – The biopen method showed improved repair of the created defect on macro- and microscopic level. In addition, early formation of hyaline-like cartilage with restoration of chondrocyte distribution and maintenance of integrity was observed after 8 weeks. However, integration of the graft with the host tissue remained problematic and high variability in regeneration was observed[61].

The main hurdle in cartilage bioprinting is to mimic the mechanical properties found *in vivo*. In native cartilage tissue, water comprises 70 – 85 % of the cartilage's total weight, whereas collagen and proteoglycans make up for 60 – 70 and ~30 % of the dry weight, respectively. When mechanical pressure is applied, the interstitial fluid flows through the ECM pores, causing a pressure gradient. In addition, the polar water interacts with the negatively charged glycans, acting as a brake on fluid displacement[62]. With bioprinting these characteristics cannot be simulated yet. *In vivo,* cartilage ECM is able to deform when pressure is applied, whereas solid bioprinted ECM in time degenerates under such pressures. Current hydrogels can be designed to closely simulate cartilage tissue organization, however, under pressure the ECM doesn't not contain the required properties withstand the force and deform.

Bone

In the field of bone tissue engineering, 3D printing has gained interest as it could potentially reconstruct a tissue with the exact geometry of an anatomical bone defect. *In vivo*, when a relatively large part of bone is missing, an inflammatory phase is initiated in which a blood clot starts the regeneration phase. Subsequently, the blood cloth will be replaced with a more fibrous, collagenous tissue, which hardens within several weeks. The months thereafter bone remodelling, a process involved in maintaining or improving bone strength, will take place[63].

Because bone is a relative hard tissue, the approaches taken differ from those in soft tissue engineering. Hydrogels offer poor mechanical properties, limiting structural support, and protection of osteogenic cells in the construct. Scaffolds have shown to play an important role in osteoconduction – inward migration of cells that induce osteogenesis – and osteoinduction – differentiation of pluripotent cells into osteogenic cells[63]. Biopolymer hydrogels offer stiffness in the kPa range, which is still a factor of million away from native bone stiffness, GPa range. By blending synthetic polymers with biopolymers, constructs can

be made biocompatible and mechanical stiffness can be increased to low range Mpa. Therefore, a common approach in bone engineering is to use scaffolds to fill the time gap between soft, premature, bone tissue to matured tissue.

Attempts most closely approaching native bone stiffness have been made by FDM printing of PLA or PCL scaffolds, with stiffness up to 244 MPa reported[64]. Scaffolds can be designed to support initial printing followed by biodegradation, or can act long-lasting. In addition, with scaffolds there is the opportunity to create porosity within the construct. Porosity can be used to enhance cell adhesion or migration, as well as increasing oxygen and nutrient supply. Porosity does however reduce mechanical strength. It is therefore important to tune the scaffold to the tissue's needs, as cells need an environment that mimics their natural ECM. With scaffolds, a factor often overlooked is linkage of the hydrogel in the scaffold with the scaffold itself. By covalently crosslinking the hydrogel with the scaffold, it increases the strength at the interface, preventing mechanical disintegration under high strains[65].

In whole bone printing, one group took an interesting approach by starting with bioprinting of hypertrophic cartilage. Daly *et al*. hypothesized *in vitro* produced cartilaginous templates with adult mesenchymal stem cells (MSCs) could *in vivo* develop to vascularized endochondral bone. This group started by implanting three bone marrow derived MSCs loaded hydrogels into nude mice and found Arg-Gly-Asp (RGD) supplemented alginate to support chondrogenesis best. Next, a PCL scaffold was designed to support the MSC-loaded bioink, which increased the compressive modulus from an average of 3.867 to 1402 kPa. *In vivo*, the PCL scaffolds showed to stimulate the development of a more hypertrophic cartilaginous tissue, characterized by higher collagen levels than the PCL free bioink. In addition, increased vascularization was observed in the hydrogel when supported by the PCL scaffold. After obtaining these results, the group used an extrusion based bioprinter which enabled co-deposition of MSC-laden bioink and the PCL filament. By printing the bioink and PCL scaffold side-by-side, bioink free channels were created. This hypertrophic cartilage construct was subcutaneously implanted for 12 weeks, which turned 24,6% of the bioprinted construct into bone[66]. Interestingly, bone formation was observed in regions where the alginate bioink had broken down, leaving space for new tissue formation.

Highlighted by Zhang *et al*., cell free 3D engineered scaffolds are not enough to induce osteogenesis. Common approaches to induce osteogenesis in 3D printed constructs is by addition of growth factors such as bone morphogenic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF). In this paper, concerns about dose-related side effects, immunogenicity, protein instability, and short half-life were shared relating to these growth factors. This group suggested two small molecules – Resveratrol (RVS) and Strontium ranelate (SrRn) – which reportedly promote bone formation, osteogenic differentiation, angiogenesis, and inhibition of osteoclastogenesis. Therefore, 3D printed PCL scaffolds with MSCs, osteoclasts, and HUVEC loaded HAMA/GelMA hydrogels were compared with and without the aforementioned small molecules. The scaffolds were placed into defects created in the mandible of rats and evaluated after 8 weeks. After 8 weeks, the scaffold with sustained release of the small molecules showed significant improvement in bone formation compared to the scaffold-only and defect-only conditions. SrRn showed to promote a dose dependent increase in osteogenic differentiation of MSCs and proliferation of HUVECs, whereas RVS promoted HUVEC migration which lead to vascular tube formation[67]. This approach differs from more conventional bone tissue engineering, which generally focuses on formation of rigid constructs. Here, the bioprinted construct functions both as a scaffold and releasing device.

All in all, the need for mechanical strength adds an additional element to bone tissue engineering. It is often opted to used scaffolds to support cell-laden hydrogels, which

accommodate cells in bone constructs. Different hydrogel compositions are still tested to find the optimal matrixes for bone tissues, as are different materials and models for scaffolds with the goal to obtain *in vivo*-like bone stiffness. These components are often tested separately, however, therefore disregarding the interaction between the hydrogel and the scaffold. Furthermore, a fundamental problem in bone tissue engineering is that none of the materials utilized for scaffold construction show optimal characteristics. Ceramic scaffolds show reasonable strength, its ability to withstand fractures is however much lower than that of bone. Scaffolds composed of metals show poor integration with the surrounding tissue due to its different stiffness, and release of ions by corrosion can cause an inflammatory response[63]. In patients, metal constructs are utilized to aid in support, but are considered a more permanent solution. Polymeric scaffolds still lack in physiological level mechanical strength, this material has most potential however, as it is highly customizable.

Concluding remarks and Future Directions

Since the start of implementing a 3D printer in a biological setting in the early 2000's, bioprinting has developed in a rapid pace. Due to its high spatiotemporal control and unmatched printing resolution bioprinting is widely recognized as one of the most, if not the most, promising techniques in tissue engineering. The wide range of bioink polymers combined with the various printing techniques has enabled its application in several fields of tissue engineering. However, an overarching problem of bioprinted tissues is the absence of a vasculature.

Vasculature is essential for integration with the host's tissue, as it enables maintenance and functionality of the transplanted tissue. Vasculature, in combination with the lymphatic system, is essential for transport of oxygen and nutrients to the cells, and removal of waste from the cells. In skin models for example, the lymphatic system mediates in inflammation and healing of damaged skin. If a tissue implant lacks a vasculature, there is no means to deliver cues like growth factors (e.g. platelet derived, insulin-like, tissue growth factors, etc.) which are important for wound repair by initiating autocrine and paracrine signalling[39]. The inclusion of such cues and other ECM components are often overlooked. An emerging strategy in bioprinting is the use of decellularized extracellular matrix (dECM) – the remaining microenvironment after removal of cells from tissues/organs – as bioink. dECM has structural features and cues such as growth factors, enzymes, and other molecules similar to that of the target tissue. This stimulates stimulate cell maturation and proliferation, and allows for better incorporation of the implant by the host's tissue. Furthermore, dECM has shown to aid in cell guidance in several tissues, polarizing cells and allowing for vessel formation. We foresee great potential for dECM bioinks, as we expect these bioinks to greatly improve bioprinted tissue integration with the host's tissue.

In more sophisticated cell co-culture models, it is often a challenge to print biomimetic tissues. An example of such complicated co-culture systems are the skin tissues. These models have different cell types in different stages of development – mature cells on the outer surface, differentiated cells in the centre and fibroblasts at the core. All these cells have different requirements in nutrition, which is a problem when printed simultaneously. A strategy, applied by some already, is to print cells in different stages of model development. This way each subsequent cell type has time to acclimate in the construct and is less affected by stress induced by changes in medium or printing of additional layers. Another important consideration revolves around the origin of the implanted cells. In order to achieve tissue-like cell densities, an *in vitro* expandable cell origin is required. Commonly used cells capable of unlimited self-renewal and differentiation are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). It is widely recognized these pluripotent cells hold great potential to regenerate or replace impaired tissue, however, the use of these cells has

not met regulatory demands yet. Challenges such as immune rejection, genomic instability, carcinogenicity, and lack of integration with the host's tissue have to be overcome[68].

The technology readiness printing whole organs, so disregarding the bio part in bioprinting, can be demonstrated by means of a liver model created by Zein *et al*[69]. Here, anatomical models of patient and donor livers were printed, which aimed to help facilitating transplant surgery. Pre-transplant CT and MRI imaging was used to obtain the dimensions and volumes of the whole liver and internal measurements of the portal vein, left- and right hepatic vein, and biliary tree. The medical images were converted to a printable model, and subsequently printed with a Polyjet 3D printer. The printed models highly mimic the native liver, with dimensional errors of <4 mm and <1.3 mm for the entire model and vascular diameters respectively. In this example, the limiting factor in resolution is the imaging, commercial CT systems have a resolution of ~0,5 mm whereas polyjet has a resolution of ~0,02 mm. A vascular error of up to 1.3 mm is too large for whole organ printing, as arterioles for example are in the range of 0,008 – 0,06 mm. Therefore, for whole organ printing also a relatively accurate technique such as polyjet falls short. However, not all transplanted tissues need vasculature down to arteriole level, rendering the current available printing techniques accurate enough to print more basic tissues. The prospect of whole organ printing is not achievable with current techniques however, in order to accommodate this both the biological and technical parts have to be improved.

Altogether, bioprinting remains to become one of the most exciting techniques in tissue engineering. *In vitro*, the high spatiotemporal control has already shown to aid in production of more *in vivo*-like tissues. To bring bioprinted tissues to the patient, however, further progress has to be made in regulation (i.e. safety, quality, efficacy, etc) and the biological aspects of bioprinting.

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