Chapter x: 3D Bioprinting For Cardiovascular Applications

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Abstract

A heart transplant is considered the gold standard treatment for end-stage heart failure patients, however the operation is reliant on organ donors and poses several clinical and economical risk factors. Tissue engineering has shown promise in providing a future alternative for organ transplants. The potential for mimicking native healthy tissue has been brought closer to realisation with 3D bioprinting. The method allows for precise deposition of cellular material into biomaterials suitable for transplantation. By culturing the cellular material using patient stem cells, the 3D bioprinted transplant can be personalised for each patient. This book chapter presents the current state of 3D bioprinting applied in developing personalised cardiac patches. An overview of cardiac anatomy and the impact of myocardial infarction is followed by a comparison of 3D bioprinting methods, commonly used biomaterials, and the characteristics of cells and cellular spheroids. This is followed by a review of findings from animal studies using 3D bioprinted cardiac patches to date and highlights the challenges of translating the method for clinical studies. Biological and design considerations for developing personalised cardiac patches conclude the review leading to the discussion of the chapter findings.

x.1 Introduction

Ischaemic heart disease is characterised by occlusion of the coronary circulation to the heart, leading to myocardial damage and death of cardiomyocytes in the impacted region.[1] This is common of myocardial infarction (MI), which may eventually lead to heart failure (HF) in more severe conditions.[1] End-stage HF is a serious condition with a poor survival rate, and the gold standard treatment option remains a heart transplant. Lack of donors, requirement for specialist surgical centres, and high cost prevents access to the treatment for a vast majority of the 26 million HF patients globally.[1]

More recently, there has been evidence for a potential alternative to replacing the whole heart by using donor transplant with a bioengineered heart tissue that combines state-of-the-art 3D bioprinting technology and stem cell-derived cardiac cells.[2, 3] 3D bioprinting technology is an adaptable method of biofabrication for the precise geometrical deposition of cells and biomaterials to create a viable and functional tissue.[3] The accuracy of the biofabricated tissue is limited by the technical specifications of the 3D bioprinting system used.[4] Being able to determine where in the engineered tissue specific cell types or non-cellular material reside is a distinct benefit compared to seeding cells and fabricating tissue in a Petri dish.[5] Similarly, 3D bioprinting, as the abbreviation suggests, makes it possible to create tissue according to 3D geometry.[4] Compared to two-dimensional shapes fabricated in a cell culture or a well plate, 3D designs are more detailed allowing complexity in the design.[4] Stem cell technologies enable the use of a patient-specific biological blueprint.[6] Latest advancements in the field have led to the use of either human embryonic or induced pluripotent stem cells (iPSCs) into cell types found in myocardial tissue.[2, 6] The benefits of using patient-derived iPSCs prevents the likely risk of transplant rejection.[5]

Cardiac tissue engineering has proven challenging due to the complex anatomy and physiology of the human heart.[3] First, emulating the complexity of the myocardial tissue using 3D bioprinting requires a perfect balance of printability and biocompatibility of the chosen biomaterials.[3] Then, from a biomechanical perspective a healthy human heart beats and contracts in a variety of directions and acts like a pump to circulate blood around the body, as well as it contracts.[7] When the heart contracts to circulate blood to the rest of the body, the top region twists clockwise while the bottom of the heart twists counter-clockwise.[7] This adds to the complexity of engineering cardiac tissue because correct mechanical properties such as rigidity and elasticity are required to accommodate this ventricular motion. For optimal myocardial tissue engineering, the engrafted cardiac tissue should contract synchronously with the native myocardium.[8] Finally, the engineered tissue should be able to engraft and ultimately improve the cardiac function of a failing heart.[2]

The myocardium is composed by three major cell types: cardiac myocytes, endothelial cells and fibroblasts.[5] The adult myocardium cannot regenerate itself following an injury.[2] Angiogenesis and biocompatibility of biomaterials used are important aspects for the successful transplantation of a biofabricated myocardial tissue. This will ensure proper neovascularization in the infarcted area for nutrient and oxygen supply, as well as the prevention of graft rejection and undesired immune response.[2]

This book chapter aims at highlighting the requirements of optimal myocardial tissues, as well as the outcomes of recent studies using biofabricated tissues for myocardial regeneration, including considerations for the translation of patient-specific, 3D bioprinted cardiac patches from exploratory studies to the clinic.

x.2 Anatomy of the Human Heart

The heart acts as the muscular pump of the circulatory system.[9] It is located in the middle mediastinum and surrounded by a pericardial sac and consists of a left and a right side divided by a septum and is formed by four distinct chambers.[9] The chamber walls have three layers: a thin internal layer of endocardium that lines the chamber walls; a thick, muscular myocardium in the middle responsible for contractions; and a thin external layer, epicardium.[9] The atrium and the ventricle on the right collect and distribute deoxygenated blood, while the same structures on the left circulate oxygenated blood.[10] The human heart has the following four exterior surfaces: anterior surface formed mainly by the right ventricle; diaphragmatic surface formed primarily by the left ventricle and partly by the right ventricle; right pulmonary surface comprising of the right atrium; and left pulmonary surface encompassing the left ventricle.[9] The heart has its own systemic blood supply, coronary circulation, which is responsible for the delivery and return of the blood flow to and from the myocardium.[9] If the coronary arteries become occluded the blood supply to the myocardial wall is compromised, which can ultimately lead to MI.

The biological function of the heart is divided in to two phases, diastole and systole, which together complete the cardiac cycle as presented in Figure 1.[11] During diastole blood is returned to the right atrium via the inferior and superior vena cava, and via pulmonary veins to the left atrium. Returning blood flow increases the pressure within the atria, and when it exceeds ventricular pressure atrioventricular valves open passively allowing blood flow into the ventricles.[11] At the start of systole atrial walls contract filling the ventricles. During a brief period of isovolumetric contraction of the ventricular walls, the atrioventricular, pulmonary, and aortic valves remain closed. As the pressure inside the ventricles increases and exceeds the systemic arterial pressure, pulmonary and aortic valves open ejecting the blood into the

circulatory system. After contracting the ventricular walls relax and the next cardiac cycle begins.[11]

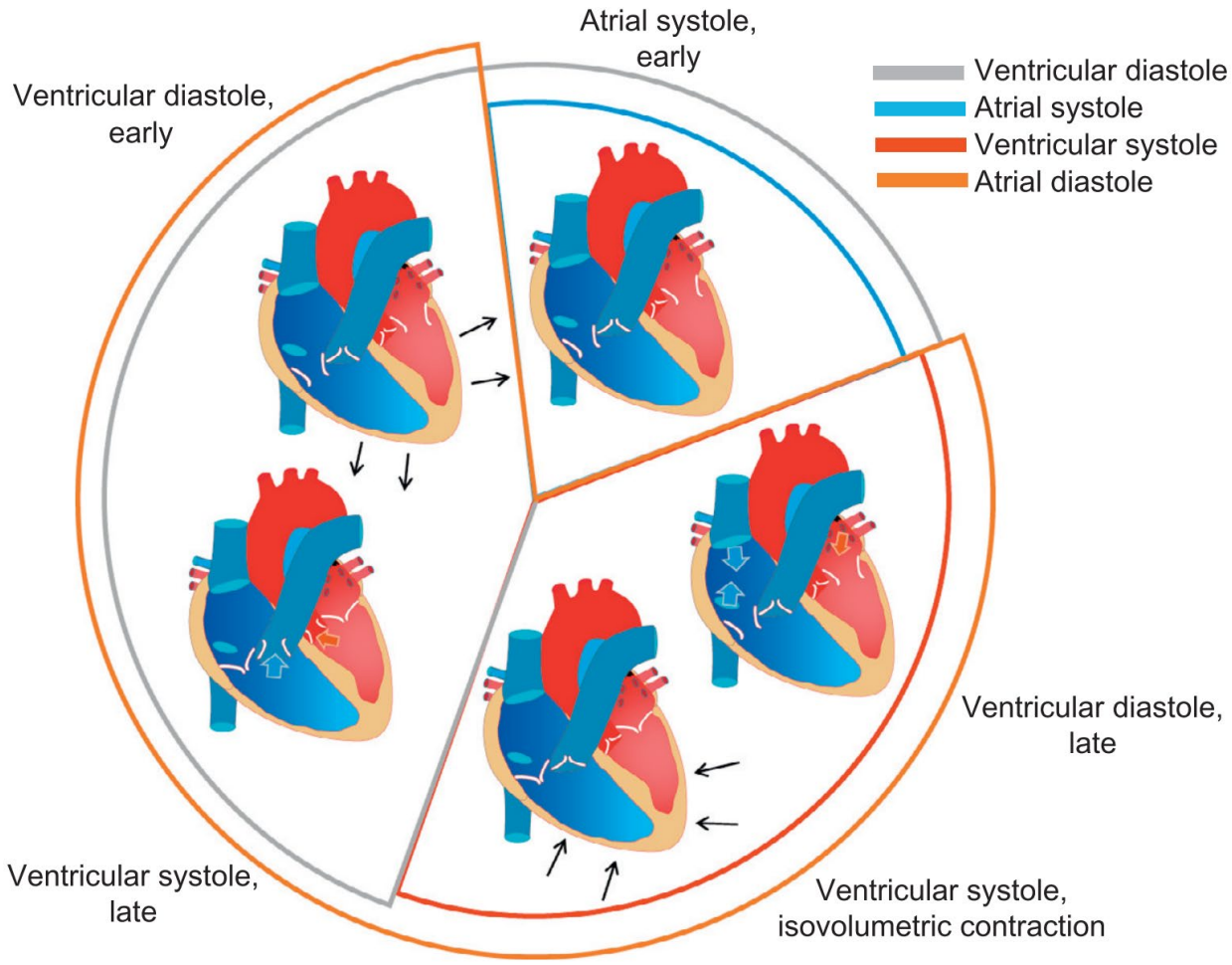

Figure 1: Phases of the cardiac cycle. Blood is collected in the atria during diastole, and flows into the ventricles and is ejected to the aorta and pulmonary trunk in systole. (Source: Athanasiou et al. 2017)[12]

x.3 Impact of MI

The sudden occlusion of the blood flow to the myocardium causes MI in the impacted region, leading to pathological cell death or necrosis.[13] The impact of MI on the myocardial wall leads to the build-up of fibrous tissue in infarcted areas. This event is known as cardiac remodelling, and results in alterations in the tissue composition, increased stiffness in the cardiac tissue,

thinning of the walls, ischaemic mitral regurgitation, and further death of cardiomyocytes.[14] The end result of cardiac remodelling is ventricular dysfunction, which leads to reduction in stroke volume and cardiac output.[14, 15] Eventually, MI can lead to HF due to the death of cardiomyocytes and fibrosis that consequently forms on the myocardial wall.[14] Despite significant advances in preventative treatments for MI, end stage HF does not have a successful cure yet.[16]

x.4 3D Bioprinting and Biofabrication Methods

3D bioprinting of cardiac cells in a hydrogel suspension may offer an alternative treatment option to individuals with myocardial damage. 3D bioprinting constitutes precise layering and positioning of cells, biomaterials and biochemicals in a defined 3D structure. This defined structure is known as a scaffold which is the structural basis for 3D bioprinting tissues. The scaffold provides a foundation for cells to adhere, proliferate and produce an extracellular matrix (ECM) to mimic living tissues.[17]

The emergence of 3D bioprinting has generated wide-spread interest and continued growth of technological advancements in the field of regenerative medicine.[18] These advancements assist in further improvement of the different techniques and methods required to successful fabricating of cardiovascular tissue structures.

Different 3D bioprinting systems operate on varying printing methods and each have their own advantages and limitations. Common 3D bioprinting methods that have been explored for bioprinting constructs for cardiovascular regeneration include extrusion-based, inkjet-based and stereolithography modalities.[19] Classification of the bioprinting methods is presented in Table 1 and illustrated in Figure 2.

Table 1: Classification of bioprinting methods, their applications, advantages, and limitations.

Bioprinting Method	Principle	Applications	Advantages	Limitations
Extrusion-based [20-22]	**Semi-solid extrusion (SSE) & Pressure-assisted bioprinting (PAB) -** rotating screw or pressurised air drives material through a loaded nozzle that deposits the material on a plane to form a desired 3D structure.	Biofabricate scaffolds that closely resemble tissue structures. Production of soft tissue models and bone structures for use as implants. Biofabrication of heart valves, blood vessels and myocardial constructs.	Capable of printing in the X, Y, Z axes. Temperature controlled printing environment. Able to use multiple nozzles to allow serial deposits.	Filament material used must transition to a stable state to preserve a three-dimensional structure
	Fused deposition modelling (FDM) - a custom temperature environment is created to melt thermo-filament materials to extrude through a nozzle that deposits on a plane to form the 3D structure.			Filament materials that require high temperatures to extrude will be detrimental to cells during bioprinting
Inkjet-based [21, 23, 24]	**Thermal inkjet bioprinting -** electrical heat is applied to the print head which creates pressure that forces droplets to release from the nozzle.	Functional skin regeneration as this technique allows for direct deposits of biomaterials onto skin Production of layered cartilage constructs	High speed allows for direct deposit and increased chances of cell viability.	Materials used to print must be in liquid state Clogged nozzles-droplet-based printing require nozzles to have small diameter outlets.

	Acoustic inkjet bioprinting - a crystal is used to convert electrical energy into mechanical energy to break the liquid material into droplets which is then deposited via the nozzle. This is the result of a piezoelectric crystal which allows for a change in elemental bonds when a voltage is applied.		Avoids thermal stress on cells such as temperature increases while bioprinting	Frequencies used may disrupt the cell membranes of bioprints. Technology is not widely available where most equipment is custom-made.
Stereolithography [20, 25, 26]	Freeform bioprinting method that does not use a nozzle to deposit materials. A computer-controlled laser, typically ultra-violet (UV), is pointed at a liquid material to solidify into a specified design. Micromirror arrays are used to adjust the light intensity of the beam to polymerise specific light-sensitive materials.	Production of tissue scaffolds and organs Fabrication of vascular networks. Biofabrication of personalised constructs for disease modelling to deliver patient specific procedures. I.e. modelling of plaque formed, calcified vessels.	Highest printing accuracy compared to the other methods. Relatively rapid printing speed. Able to produce large scaffolds. Able to fabricate complex and intricate geometrical designs.	Laser light source may affect DNA structure of autologous materials used to print.

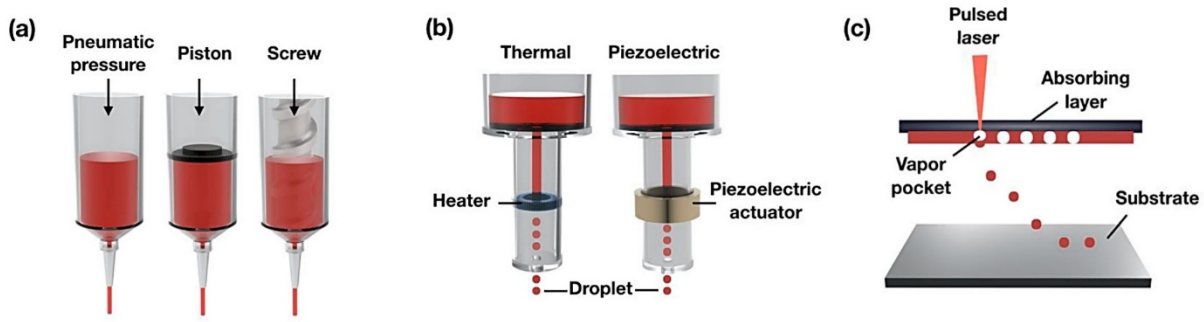

Figure 2: Major bioprinting methods. Schematic of (A) extrusion-based; (B) inkjet-based; and (C) stereolithography bioprinting systems. (Source: Fatimi et al. 2022)[27]

x.5 Bioinks

A bioink is commonly composed of a mixture of biomaterials and desired cell types for bioprinting tissue. Two dominant types of bioink structures are currently used: scaffold based bioinks consist of encapsulated cells in biomaterials, while cell-free bioinks are used for cells to adhere to them in a second time.[28] Selection of biomaterials for formulating bioinks must demonstrate biocompatibility and printability, and exhibit desired rheological properties required for the intended 3D bioprinting technique and application.[28] These characteristics are important as the material used to 3D bioprint must facilitate cell adhesion, differentiation, and proliferation.[28] These in turn determine cell viability and the ability of the biofabricated tissue to support living cells after transplantation.

A challenge in 3D bioprinting is the need to yield a robust structure where embedded cells can proliferate and adhere. A soft structure provides a softer matrix environment which is favorable for cell viability of soft tissues, while a stiffer structure may be suitable for other tissues and organs.[29] To bridge tissue-specific requirements, crosslinking strategies have been applied to polymers in low-viscosity bioinks for 3D bioprinting solid-like constructs. *In situ* and post-crosslinking approaches have both been explored, demonstrating high cell viability and improved printability *via* enhanced bioink viscosity.[29]

x.6 Hydrogel Composition

Hydrogels have been heavily explored in 3D bioprinting of cardiac patches as they are able to support cell growth and adhesion, and have tunable mechanical characteristics.[30] In addition, hydrogels provide mechanical support for cardiac cells and allow the deposition of cell-laden matrices to bio-fabricate a cardiac patch. Use of hydrogels formulated from natural polymers is common as they demonstrate good biocompatibility. However, synthetic hydrogels are easily produced compared to natural bioinks and often meet the required specifications of stability and reproducibility in 3D bioprinting.[31] Therefore, to meet mechanical properties and biocompatibility, a combination of natural and synthetic polymers is commonly used to form a hybrid hydrogel. Depending on the polymer properties, bioinks have been formulated to achieve optimal printability and cell-to-cell function. Commonly used natural bioinks are discussed below and presented in Figure 3.

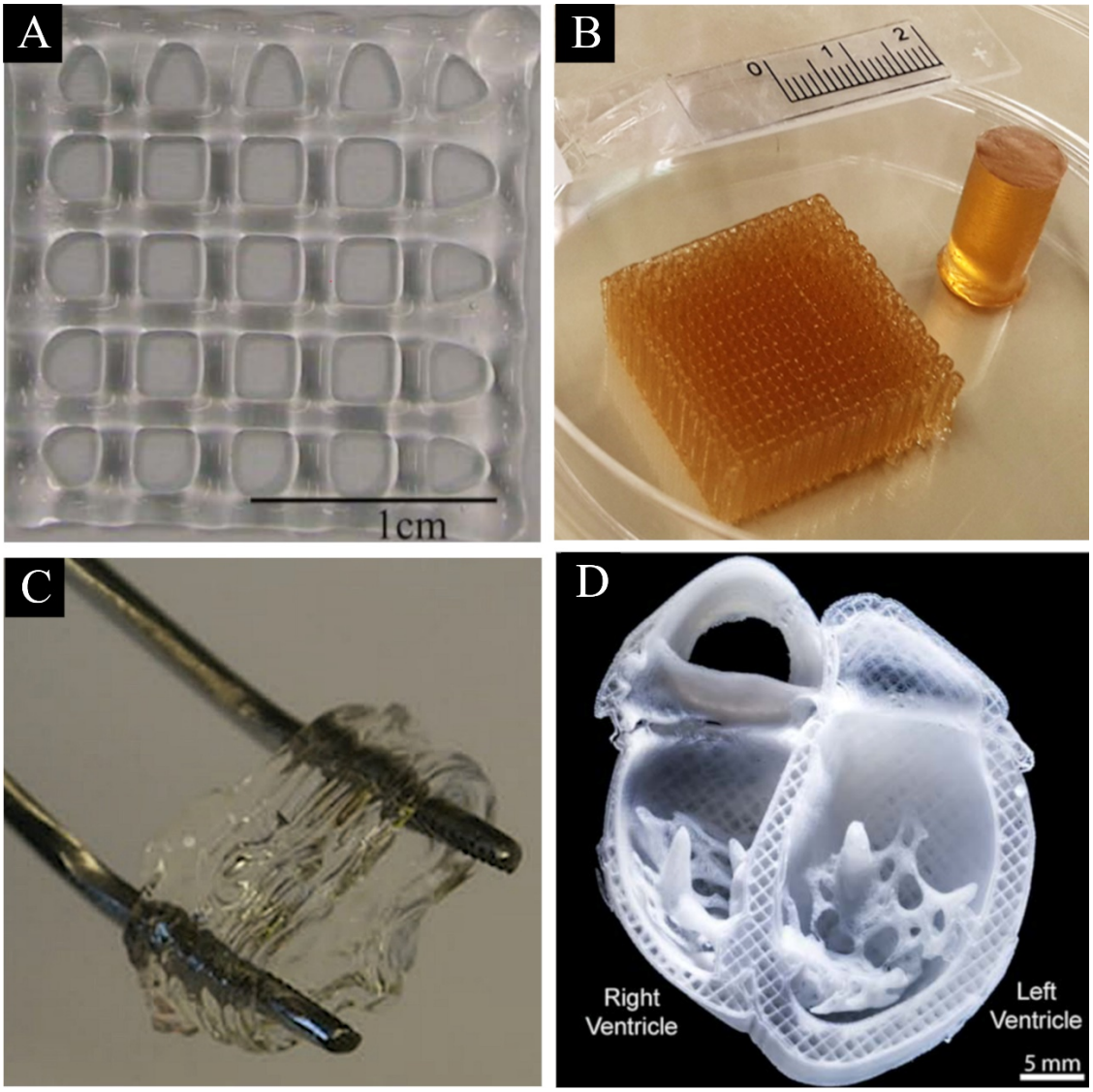

Figure 3: Examples of scaffolds containing hydrogels used to bioprint tissues. 3D bioprinted hydrogel scaffolds using (A) alginate; (B) gelatin; (C) gelatin methacrylate (GelMA); and (D) collagen. (Adapted from: Li et al. 2016; Naghieh et al. 2019; Ouyang et al. 2017; Lee et al. 2019)[32-35]

x.6.1 Alginate

Derived from brown algae, alginate exhibits water-soluble properties that are suitable for preparing hydrogels.[36] An alginate solution can be crosslinked with calcium chloride to obtain a gelatinous structure (Figure 3A). Alginate is highly abundant and cost effective resulting in its widespread use in regenerative medicine such as in 3D bioengineering tissue. Furthermore, it is non-toxic and has been shown to not cause an inflammatory response *in vivo*.[36]

x.6.2 Collagen

Collagen has been explored as a natural polymer for 3D bioprinting as it is a common protein that facilitates structural integrity and mechanical support in mammalian tissues (Figure 3B). Collagen for 3D bioprinting applications can be derived from a variety of sources, including bovine, porcine, and fish skin.[37] The insoluble nature of collagen requires an acidic solvent to transform it into a hydrogel for use as a bioink.[36] A disadvantage of 3D bioprinting with collagen is the requirement for a long crosslinking process and the material's lack of stiffness. This limits the applications of collagen when 3D bioprinting of bulky or complex structures.

x.6.3 Gelatin

Gelatin is commonly used as a bioink in 3D bioprinting due to its gelation reversibility, processability and minimal immune response during surgical application. Gelatin is sourced from Type I collagen. While collagen is an insoluble protein, gelatin can dissolve in water in temperatures over 30°C due to its thermal denaturation. The process is reversible as gelatin can undergo gelation at 4°C and become a solution at 37°C.[36] Due to this gelation property, crosslinking of gelatin is required using chemicals such as aldehyde derivatives to ensure structural integrity during 3D bioprinting (Figure 3C).[38]

x.6.4 Gelatin Methacrylate

Gelatin methacrylate (GelMa) is a common fabricated gelatin-based ink used for 3D bioprinting complex structures (Figure 3D). It is also used as an additive in bioinks to enhance mechanical properties and printability. GelMa is a product of the reaction between gelatin and methacryclic anhydride. When photocured into a hydrogel with a photoinitiator, GelMa

exhibits essential properties of an ECM to promote cell growth and proliferation, which allows cells to populate the scaffold.[39]

x.7 Cell Types Used in Bioinks

Cardiac monocytes and nonmyocytes, such as endothelial cells and fibroblasts, constitute approximately 70% of the cells in the human heart.[40] Along with vascular cells and blood cells in the heart, they are vital in providing structural integrity and electrical stimulation to the myocardium.

Cells used in 3D bioprinting can be autologous or allogeneic, to date both have been investigated for use in cardiac regeneration. These cells are mixed with various polymers and structures to formulate a bioink used in 3D bioprinting. Multipotent and pluripotent stem cells have been explored for 3D bioprinting cardiac patches to restore cardiomyocytes and vascular networks in myocardial infarcted areas. Multipotent stem cells can be sourced from peripheral blood, bone marrow and living tissue. This cell type has been reported to possess cardiogenic differentiation capabilities which improves the cardiac patch cell viability.[41] Pluripotent stem cells can be sourced from embryonic and induced pluripotent stem cells. This cell type is highly attractive for the use in cardiac regeneration due to its ability to self-renew and multilineage differentiation. Embryonic stem cell-derived cardiomyocytes (ESC-CMs) have been reported to be able to electromechanically couple with the host cells.[41] This allows for simultaneous contraction between the host's heart and the implanted cardiomyocytes.

x.8 Spheroids for 3D Bioprinting

For successful biomimicry and biocompatibility of forming complex engineered tissue, cell-to-cell contact is essential for intracellular functions. Surrounding the intracellular region is the ECM that supports cell differentiation, homeostasis, and cell proliferation.[42]

Cells in the heart are surrounded and subjected to physiological stimulations such as other mass components interfering spatial distributions of oxygen, nutrients and signaling molecules.[43] This is important to consider when 3D bioprinting engineered tissues as the vascular network is critical since the transplantation of the tissue to support healthy transportation of nutrient and oxygen supplies. To mimic these physiological conditions in 3D bioprinted tissues and to achieve optimal biomimicry and biocompatibility, the use of spheroids has been explored as building blocks for tissue engineering.[42]

Spheroids are generated to facilitate cell-to-cell interactions while maintaining cell-matrix adhesion. Spheroids harness unique properties which make them advantageous for tissue engineering applications. When compared to a 2D single cell approach, spheroids exhibit greater regenerative properties.[42] Complex tissues can be biofabricated with spheroids as incorporating multiple cell types in the same media is possible with co-culture spheroids. In addition, spheroids can be manipulated into macrotissue constructs for the possibility of engineering large tissue constructs. Pairs of uniluminal vascular spheroids in cultured medium have been investigated and found to form larger spheroid structures *via* self-assembly.[44] This potentially allows for the use of vascular spheroids to be the building blocks to 3D bioprint blood vessels.

It is important to highlight that there are limitations with current *in vitro* models in 3D bioprinting and the methods of using a single layer of cells to 3D bioprint. A significant limitation of this is that the cell growth remains linear, known as “2D cultures”, which impacts

transplantation on the natural three-dimensional structure of the tissue.[45] It has been researched that 3D cultures have an advantage of having better biomimicry *in vivo*. *In vitro*, 3D cultures can better mimic the cardiac tissue microenvironment which assists in improving the accuracy of *in vitro* and *in vivo* testing. A major 3D culture type are spheroids. Spheroid cultures have been used to 3D bioprint tissue and organ models.[46]

More specifically, cardiac spheroids have been investigated with human patient derived cardiomyocytes in a range of culture and 3D bioprinting techniques. Using cardiac spheroids to biofabricate cardiac tissue offers increased potential for improved cell-cell interactions, vascularization via coculture of fibroblast-based cells and endothelial cells, and the production of ECM via the spheroid's nature of self-assembly.[45]

Techniques when 3D bioprinting with spheroids include scaffold-free where spheroids are suspended in media, and scaffold-based where the spheroids are embedded in a hydrogel and then printed. A cardiac spheroid, alginate-gelatin based hydrogel was reported to enable fusion of the spheroids which resulted in the cardiac spheroids exhibiting contractions when stimulated.[46] This highlights the long-term use of cardiac spheroids potentially being used to biofabricate cardiac tissues. Another study explored the biofabrication of myocardium layers with isolated, autologous cardiac progenitor cells (CPCs) to form cardiac spheroids. These CPCs-based spheroids found to 3D bioprint ideal gelatin and collagen scaffolds to regenerated damaged myocardium.[47]

x.9 Considerations for Testing of Regenerative Properties of 3D Bioprinted Tissues in Preclinical and Clinical Studies

Several different small and large animal models have been used in cardiac research to date. Selecting an animal model that is the best fit for the study design depends on the scientific

hypothesis of the experiment, availability of resources for managing the animals for the duration of the study, and how well the anatomical features under investigation match human anatomy.[48]

Rats and mice are commonly used small animal models in cardiac studies as they exhibit similar coronary circulation architecture to humans.[48, 49] Canine, ovine, and porcine models have been used in large animal studies investigating the mechanism of MI.[48, 50] It is important to highlight that the heart of a pig is anatomically and physiologically closest to its human counterpart. The anatomical position, shape, average rest heart rate, weight ratio to body weight, and thickness of the left ventricular (LV) wall in humans, pigs, rats, and mice are presented for comparison in Figure 4.

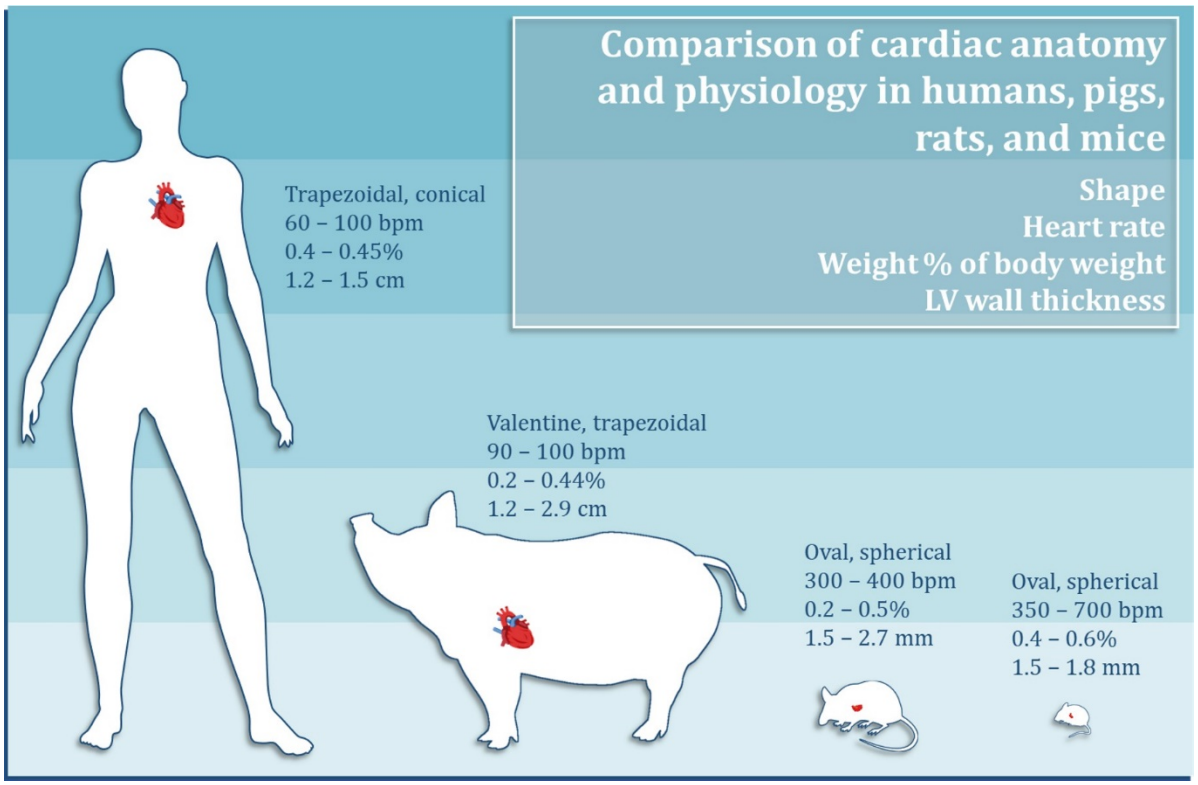

Figure 4: Comparison of cardiac anatomy and physiology between humans, pigs, rats, and mice. The anatomical size and orientation of the heart is similar in humans and pigs, but notably different when compared to rodents. In contrast the comparative size of the heart to body weight and thickness of the LV wall are similar across all species.

The anatomical orientation and physiology of the heart in small and large animals differs to that of humans. In rodents, the heart is significantly smaller, has a more rounded shape, and the apex of the heart points caudally. Residing in a pericardial sack in the mediastinum, the rodent heart does not have a ligament attachment to the diaphragm allowing it to maintain a spherical silhouette.[49]

In large quadruped animals such as pigs the size of the heart is similar to bipedal humans, but the anatomical positioning and overall shape of the organ are different.[49, 51, 52] The porcine heart is more elongated with a valentine shape compared to the human heart. In pigs, the heart rests on its apex with the anterior border near the sternum, while the flat inferior border of the human heart rests on the diaphragmatic surface giving the organ a trapezoidal shape. In both species the heart is firmly attached to the central tendinous aponeurosis of the diaphragm.

A handful of clinical studies have so far been conducted where stem cell therapies were tested for the treatment of MI. As a comparison to other approaches, none are yet to use a 3D bioprinted cardiac patch in humans, but have instead tested cell sheets[53-56] and injectable stem cell treatments.[57-60] Autologous cell sheets formed of skeletal myoblasts[54-56], and hESC-derived cardiovascular progenitors embedded in a fibrin patch[53] are the closest experimental methods to a 3D bioprinted cardiac patch tested clinically to date.

Although the clinical studies using a cell sheet approach recruited small cohorts between 1 to 7 patients, results showed promising trends in the areas of efficacy, feasibility and safety.[53-56] In terms of efficacy, all the clinical studies saw a trend in improvement of the LV ejection fraction (LVEF) with increases ranging between 7.1% and 20%, however none of the results were statistically significant.[53-56] Two of the clinical studies measured local changes to the LV wall motion in the treated area.[53, 56] Menasché et al.[53] used a scoring system for the LV wall motion measurement and reported a significant change from 4.2 ± 0.8 at baseline to 2.5 ± 0.4

at 1 year where a lower number indicates an improved outcome. Yoshikawa et al.[56] measured systolic wall motion in the region of transplanted cell sheets and recorded a change from 4.6 ± 1.0 mm to 5.0 ± 1.4 mm after the treatment, however the change was not significant.

Two clinical studies discussed the better safety and feasibility of the cell sheet method over injectables.[55, 56] Main benefits cited were improved cell delivery to the transplant site, increased cell survivability, reduced risk of inflammation in the myocardium, and reduced myocardial damage. None of the clinical studies encountered major or severe adverse events such as severe arrhythmia, post-procedural MI, or patient death attributed to a cell sheet transplant, or reported on rejection of the cellular transplant.[53-56]

x.9.3 Studies Using 3D Bioprinted Cardiac Patches in Animal Models

To date, a handful of *in vivo* studies investigating the use of 3D bioprinted cardiac patches for the treatment of MI have been conducted. Six small animal studies where a 3D bioprinted patch has been transplanted to the left ventricular wall were completed between 2011 and 2022.[61-66] Findings of studies where a 3D bioprinted cardiac patch was used are presented in Table 2. Two of the studies were done on mice and four on rats, so far studies of 3DBP cardiac patches have not been done on large animal models. In general, the results indicate a trend in improvement in the ejection fraction and fraction shortening, reduction in MI size, and neovascularisation after patch implantation.

Four out of the six studies testing a bioprinted patch *in vivo* used a permanent left anterior descending artery (LAD) ligation model, one an ischemic reperfusion (I/R) model, and in one study MI was not induced. Engraftment to the epicardium ranged from freeform transplantation to the use of sutures, fibrin glue, or a combination of both.

Duration of the studies ranged between 7 to 120 days, where available results of cardiac performance measurements are included at 28 days post-MI in Table 2. As the studies were designed and performed independently, measurement times and end points, size of control and treatment groups, as well as cardiac and patch performance indicators vary between the studies making them difficult to contrast and compare.

For cardiac performance, ejection fraction (EF) was the most commonly recorded indicator and reported in five of the six studies.[61-64, 66] In only three studies the bioprinted patch increased the EF% significantly compared to the control group of animals with MI[61, 64, 66], the other two studies[62, 63] reported a trend towards improved EF but the findings were not significant. Further to this, in two of the studies the bioengineered cardiac patch significantly increased fractional shortening % (FS%) post-MI when compared to a control group of mice with MI only[61], or when comparing performance to a treatment group with an acellular patch.[64] Cardiac output (CO) was also recorded in two of the studies but reported non-significant results between 4.55 mL/min and 36 mL/min.[61, 63]

Adhesion and engraftment of the cardiac patch to the transplantation site was reported in four of the studies, and confirmed at the end of the study when the animals were euthanised.[61, 62, 65, 66] No difference was found between different engraftment methods in terms of the patch remaining in place, cellular engraftment between the cardiac patch and native myocardium was tested histologically and confirmed in three of the studies.[62, 65, 66]

Changes to the LV morphology were measured using varying methods and different parameters across all studies in Table 2, including *in vivo* using echocardiography and in histology studies after hearts had been excised. None of the studies reported adverse cardiac events post-MI, such as teratoma formation, alloimmunisation, or inflammation related to a cardiac patch transplant. It should be noted however, that only one of the studies included the survival rate

of animals with 100% survival in the cardiac patch treatment group and 83.3% survival rate in the control group.[63]

Histological analyses demonstrated a significant decrease in the fibrotic area in three studies[61, 63, 64], and non-significant results in two studies[62, 66] after a cardiac patch implantation, when compared to control animals with MI only. Immunostaining was used to quantify markers for cardiomyogenesis and angiogenesis in the transplanted area, as a promising sign of cardiac regeneration. Although two studies had statistically non-significant results[61, 65], four studies saw a significant increase in a number and density of the vascularity compared to acellular controls.[62-64, 66] In addition, cardiomyocyte proliferation was measured in two studies: a significant increase in the number of cardiomyocytes between an acellular patch group and the cardiac patch group post-MI was reported in one study[61], while the second returned a non-significant result.[62]

Table 2: Studies testing 3D bioprinted cardiac patches *in vivo*.

Study	Hydrogel composition	Cells (type, ratio, #)	Patch geometry (layers, thickness (mm), diameter (mm))	Animal / MI model	Suture / glue	Measurement timing post-MI (d)	EF% (n) (Sham, MI only, acellular patch, random cell-seed, engineered patch)	FS% (n) (Sham, MI only, acellular patch, random cell-seed, engineered patch)	MI size (n) (Sham, MI only, acellular patch, random cell-seed, engineered patch)	Graft presence (macro / micro scale)	Vascularisation (n)
Jiang et al., 2022[61]	ASP-A35m	CMs, neonatal mice N/A N/A	2 1 5	ICR mouse, permanent	None	28	55.58 ± 2.62 (7) 22.49 ± 1.67 (7) 23.33 ± 1.49 (7) - 29.31 ± 7.32# (7)	28.57 ± 1.37 (7) 10.27 ± 0.78 (7) 10.74 ± 0.78 (7) - 13.83 ± 3.75# (7)	- ns. (7) ns. (7) - Significant# (7)	Macro	ns. (-)
Cui et al., 2020[62]	GelMA / PEGDA	hiPSC-CMs, hMSCs, hECs 1:1:1 N/A	2, 4, 8 0.6 4	NSG mouse, I/R	None	120	- 56.1 ± 1.5 (-) 64.1 ± 3.5 (-) 64.1 ± 3.5 (-) -	- - - - -	- 8.4 ± 1.1% (-) 3.8 ± 0.7% (-) 3.8 ± 0.7% (-) -	Macro, micro	Significant** (≥6)
Yang et al., 2019[64]	PGS-PCL	CMs, H9c2 rat N/A 10 x 10^4/well	4, 15 1.2, 4.5 2	Sprague-Dawley rat, permanent	Suture	28	ns. (8) ns. (8) ns.† (8) ns.‡ (8) Significant** (8)	ns. (8) ns. (8) ns.† (8) ns.‡ (8) Significant** (8)	- ns. (8) ns.† (8) ns.‡ (8) Significant** (8)	-	Significant** (8)
Yeung et al., 2019[63]	Cardiac spheroid	hiPSC-CMs, FBs, HUVECs 70:15:15 3.3 x 10^4/well	1 0.35 - 0.40 3.6	Lewis nude rat, permanent	Suture, glue	28	- - 40.1 ± 14.4 (6) 50.0 ± 18.9 (6) -	- - - - -	- - 19.39 ± 8.1% (6) 10.6 ± 5.1%* (6) -	-	Significant*** (6)
Ong et al., 2017[65]	Cardiac spheroid	hiPSC-CMs, FBs, HUVECs 70:15:15 3.3 x 10^4/CS	1 - -	Rowett nude rat, none	Suture, glue	7	- - - - -	- - - - -	- - - - -	Macro, micro	ns. (-)
Gaebel et al., 2011[66]	Polyester urethane urea	HUVECs, hMSCs 3:1 18 x 10^4/cm2	2 0.3 8	Rowett nude rat, permanent	Suture	56	ns. (4) ns. (3) ns. (6) ns. (6) Significant* (7)	- - - - -	ns. (3) ns. (3) ns. (3) ns. (3) ns. (3)	Macro, micro	Significant* (4)

All values mean ± SD, ns. non-significant, * p<0.05, ** p<0.01, *** p<0.001, # p<0.0001. † cellular cardiac patch with a PCL scaffold, ‡ cellular cardiac patch with a PGS scaffold.
ASP-A35m: leucine zipper-based protein bi-layer hydrogel, CM: cardiomyocyte, EF: ejection fraction, FB: fibroblast, FS: fraction shortening; GelMA: gelatin methacrylate, hEC: human endothelial cell, hiPSC: human induced pluripotent stem cell, hMSC: human mesenchymal stem cell, HUVEC: human umbilical vein endothelial cell, ICR: Institute of Cancer Research, I/R: ischemic reperfusion, MI: myocardial infarction, NSG: NOD scid gamma, PCL: poly-(ε-caprolactone), PEGDA: polyethylene glycol diacrylate, PGS: poly-(glycerol sebacate).

x.10 Considerations for the Development of Personalised Cardiac Patches

Personalised medicine aims at transforming the standardised healthcare approach to treatments tailored to suit the needs of an individual patient. 3D bioprinting is an ideal method of biofabrication for this purpose, as in wider terms 3D printing is considered perfect for producing bespoke solutions. To 3D bioprint a patient-specific transplant the cardiac patch must first be designed to match the patient's genetic and anatomical cardiac profile.

Elements of designing and 3D bioprinting a patient-specific cardiac patch have been described in our previously published review.[1] The process is presented in Figure 5, where the left side represents the biological components of the design and the right side considerations regarding the anatomical 3D modelling.

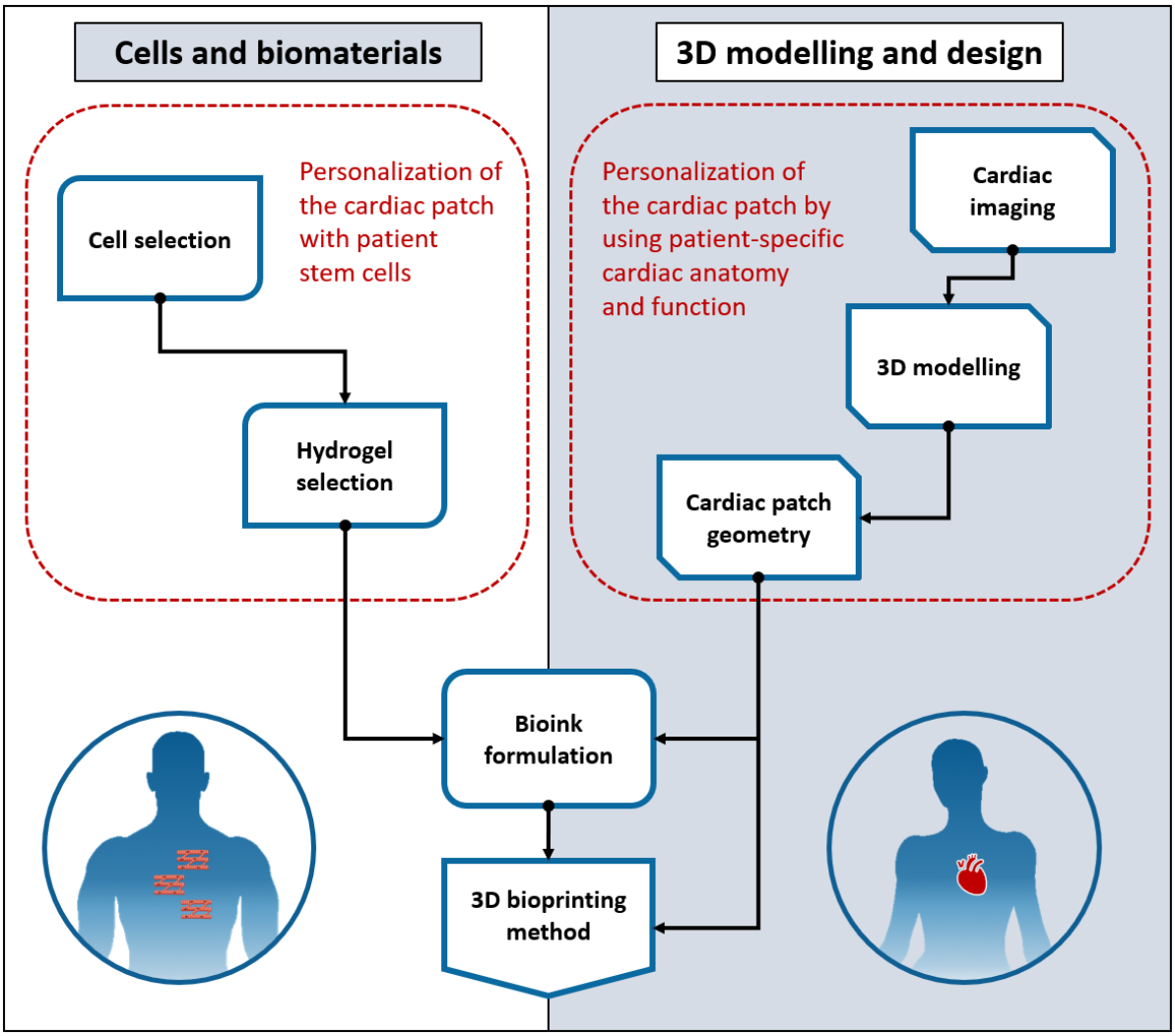

Figure 5: Biological and design considerations of creating a patient-specific cardiac patch. Selection of the cell line and hydrogel together with the 3D design of the cardiac patch determine optimal bioink formulation and 3D bioprinting method. (Source: Matthews et al. 2022)[1]

As previously discussed, to tailor the biological basis of the cardiac patch to match individual patients, patient stem cells are reprogrammed to provide the cellular content of the biofabricated cardiac tissue. An autologous approach to the biological design is expected to carry a smaller risk of transplant rejection as the cells originate from the patient. If a hydrogel is used in the construction of the cardiac patch, the material has to support cell viability, ensure the maturation of the cells, as well as maintain cell expression.

Cardiac anatomy and function are typically measured by an echocardiogram, cardiac computed tomography (CT), or cardiac magnetic resonance imaging (MRI). Each imaging modality has its benefits and ideal use. For the purpose of 3D modelling and design, CT scans are used to capture the anatomical features of the heart while MRI identifies the location and severity of MI in the LV wall. Data from the cardiac imaging is segmented using specialized software which produces a detailed digital 3D model of the anatomical features. A cardiac patch that conforms to the patient anatomy in the infarcted region is designed using the digital model, typically with a CAD software which produces a file suitable for 3D bioprinting. The concept of the process is presented in Figure 6.

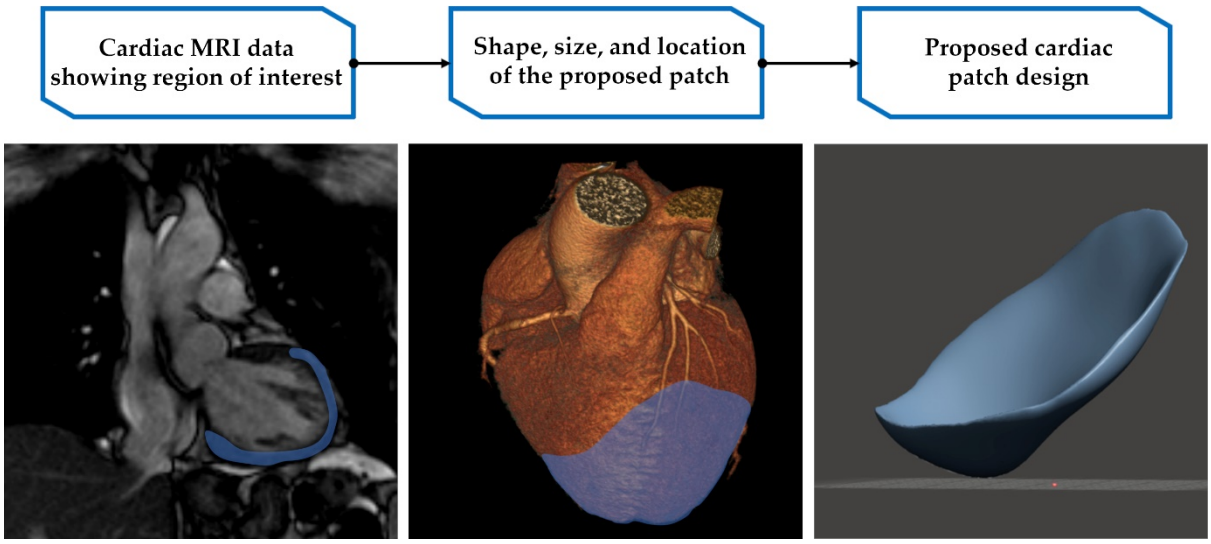

Figure 6: 3D design of personalised cardiac patches using MRI and CT scan data. The infarcted region in the myocardium is identified in a cardiac MRI, and a cardiac CT scan captures the anatomy of the LV wall. Data from the scans is used to create a digital 3D model of a patient-specific cardiac patch. (Source: Matthews et al. 2022)[1]

Properties of the selected hydrogel and the overall cardiac patch geometry form a basis for the bioink formulation, as it must combine optimal biological and mechanical properties. Biological traits such as biomimicry, cytocompatibility, and rate of degradation are required to maintain cell survivability within the patch. Mechanical properties including printability,

rheology and swelling behaviour impact the biofabrication of the cardiac patch to a patient-specific 3D geometry. Finally, the bioink composition and the 3D design determine the most favourable 3D bioprinting method. The modality must allow for producing the personalized cardiac patch using the selected biomaterials, and be suitable for 3D bioprinting the patch according to its shape and dimensions.

x.11 Discussion

Despite several *in vitro* and *in vivo* preclinical studies, a 3D bioprinted cardiac patch has not been transplanted to a patient's heart. Clinical studies using stem cells for the treatment of MI have so far either utilised cell sheet technology, or stem cells were delivered to the myocardium using an injection. In general, studies conducted to date have been early phase studies that do not provide enough evidence for regulatory approval.

Adapting the method of biofabricating and transplanting a cardiac patch from an animal model to a human study presents several challenges. While several studies have been conducted to date to the use of stem cell transplants for treatment of MI, research protocols were designed independently using a variety of methods and materials. In general, standardised test protocols should be developed for assessing the efficacy, feasibility, and safety of any type of cardiac patch regardless of the biofabrication method, bioink formulation, or cellular/acellular composition.[4, 67] Without standardised test protocols results will remain difficult to compare limiting their usefulness in future studies. There is, therefore, a definite need for better direction in the field of 3D bioprinting in terms of finding best practices to ensure fast and safe translation[4, 45, 67].

Although studies using 3D bioprinted cardiac patches have already been conducted successfully in small animals, there is still more research to be done before translation to a

clinical solution can be achieved. For instance, anatomical and physiological differences to humans are evident as shown in Figure 4. Due to the poor comparability, results from small animal models are generally not enough to meet regulatory approval required for progressing to clinical studies.[51] Compared to small animal models, studies using large animals such as pigs have higher resource requirements for carrying out experiments and caring for the animals for the duration of the study. Adding to this, although the anatomical features of the heart are similar between pigs and humans, the age of the test animal is another point of consideration. In juvenile pigs the growth rate of the animal has to be incorporated to long-term plans as this is likely to result in changes to the myocardial wall.[48] An even bigger obstacle however is the lack of established protocols for generating stable pluripotent stem cells for using pigs in cardiac patch studies.[2]

All *in-vivo* studies discussed in this chapter, including the large animal and clinical studies where 3D bioprinting was not used, had very small sample sizes.[53-56, 61-66] The exception is the clinical study by Menasché et al.[58] where a cohort of 120 patients was recruited and the study was conducted in 21 university hospitals across 5 countries. Despite of the investment in resources and cross-industry collaboration, the cohort was reduced to 97 patients to fit the study criteria, and the small sample size was reported as a limitation impacting statistical significance of the findings.[58]

The enormous effort required for conducting clinically and statistically relevant studies leads to a substantial problem when designing future large animal and clinical studies for 3D bioprinted cardiac patch transplantation. For studies using a large animal model with a statistically significant cohort, the appropriate number of test animals and research facilities will be expensive to obtain.[50] Further to this, recruiting patients for a clinical study of a similar magnitude would call for a multicentre and multidisciplinary approach.[68] Such considerations

are difficult to resolve. They are however necessary to overcome if the method of using 3D bioprinted cardiac patches is to progress to phase I and phase II clinical trials.

In recent years the materials and methods used in 3D bioprinting have developed significantly. The technology and materials for producing 3D bioprinted cardiac patches are available, but studies undertaken to date are yet to utilise these advances to their full extent. We have presented here a concept for the process of personalising the cardiac patch according to patient biology and cardiac anatomy. A distinct advantage of any 3D printing modality is the potential of prototyping a bespoke design which is ideal for personalised medicine where a patient-specific treatment is the main goal. Medical imaging together with modern design software allow creating personalised cardiac patches as 3D bioprinters use file formats commonly produced by CAD software.

As long as cardiovascular disease (CVD) persists as the primary cause of death globally, medical research will look for alternatives for the treatment of CVD and HF. As described in this book chapter, current lack of consistency in 3D bioprinted cardiac patch studies provides an opportunity to establish clinically relevant approaches to facilitate their testing and translation to the clinic.

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