Investigating a novel intramyocardial delivery method for induced pluripotent stem cell-derived cardiomyocytes

Thesis submitted for the degree of Doctor of Philosophy
University College London

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**Declaration**

‘I, Annalisa Bettini confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

Signature:  
Date:
Acknowledgements

I am sincerely thankful to my primary supervisor Professor Richard Day for his constant guidance and motivation. His everyday support encouraged and pushed me to grow into the scientist that I am today. Likewise, I am immensely thankful to my secondary supervisor Dr. Daniel Stuckey for continuously advising, supporting and listening. His enthusiasm pushed my research and thinking further than I could have imagined. Thank you both for believing in me and inspiring me, I could not have asked for better advisors.

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Abstract

Cell therapy is a potential novel treatment for cardiac regeneration and numerous studies have attempted to transplant cells to regenerate the myocardium lost during myocardial infarction. To date, only minimal improvements to cardiac function have been reported. This is likely to occur from low cell retention following delivery and high cell death after transplantation.

The thesis aimed to improve the delivery and engraftment of viable cells by using an injectable biomaterial which provides an implantable, biodegradable substrate for attachment and growth of cardiomyocytes derived from induced pluripotent stem cells (iPSC).

The thesis describes the fabrication and characterisation of Thermally Induced Phase Separation (TIPS) microspheres, and functionalisation of the microspheres to enable cell attachment in xeno-free conditions. The selected formulation resulted in iPSC attachment, expansion, and retention of pluripotent phenotype.

Differentiation of iPSC into cardiomyocytes was investigated and characterised, comparing in vitro culture to microsphere culture using flow cytometry, immunocytochemistry and western blotting techniques. Microsphere culture was shown to be protective against anoikis and compatible for injectable delivery.

The in vivo compatibility of the microspheres was assessed using pre-clinical murine models. The microspheres were rendered trackable, using the computed tomography contrast agent barium sulphate, to assess the distribution after ultrasound guided intramyocardial injections for targeted delivery. The findings suggest that barium sulphate-loaded microspheres can be used as a novel tool for optimising delivery techniques and tracking persistence and distribution of implanted products. Once in vivo compatibility was established, a cellularised microsphere formulation was delivered to the myocardium of immunocompromised mice, to compare the efficacy of biomaterial assisted versus suspension cell therapy.

This work demonstrates that TIPS microcarriers offer a supporting matrix for culturing iPSC and iPSC derived cardiomyocytes in vitro and when implanted in vivo have the potential to be developed into an injectable biomaterial for cardiac regeneration.
Impact Statement

Myocardial infarction leading to chronic heart failure (CHF) is a major healthcare burden, with an estimated 900,000 people suffering from the condition and 60,000 new cases yearly. The NHS spends 1–2% of its annual budget managing the condition, costing around £625 million. Treatment exists to manage the symptoms, but there is no cure available. Hence, there has been an increasing interest in the use of cell-based therapy to provide a solution for CHF.

Over the past 20 years, numerous clinical trials have attempted to deliver various cell types to regenerate damaged heart tissue. Despite some studies showing beneficial effects including improvement of ventricular function, the results have generally been underwhelming. Poor cell retention and engraftment is one of the main factors attributed to the response observed.

Biomaterials have been used to improve cell delivery and survival; however, they are most often delivered in an invasive manner. After administration therapies are not further tracked, missing an opportunity to further optimise the therapy. Therefore, development of new treatment modalities is an important field of research.

This thesis focused on expanding our understanding of induced pluripotent stem cell (iPSC)-based cell therapies for cardiac regeneration and in particular, the use of a microcarrier developed by our group to assist the therapeutic potential of cell therapy. The findings presented in this thesis explore and present Thermally Induced Phase Separation (TIPS) microspheres as an injectable cell substrate to improve cell retention and engraftment of live cells. The data presents a novel application for the TIPS microspheres, and new methods to prime cell attachment under xeno-free conditions. The formulation of trackable microspheres provides a novel tool for optimising delivery techniques and tracking persistence and distribution of implanted products. Not only is this important in understanding the design of a clinical product, but it also has the potential to pave the way for image guided, minimally invasive therapies in the future.

The tools developed for the study of iPSC and iPSC derived cardiomyocytes (iPSC-CM) would also be useful to any investigations assessing iPSC-based cell therapies. In this study, flow cytometry panels for the assessment of anoikis, cardiac and
pluripotent cell phenotype were optimised. Calcium imaging was used to assess iPSC-CM contractile functionality on the microsphere construct, and this technique has never been used on a cell-laden microcarrier. In addition, the comprehensive qPCR-based method developed here will allow further monitoring of trilineage differentiation of iPSC by biomaterial modulation. These tools optimised can contribute to research methodologies and either yield or answer more unknowns.

The outputs of this thesis have been disseminated through public engagement activities, internal and external collaborations, and presentations at national and international conferences.

Overall, this work furthers our understanding of iPSC, iPSC-derived products, and biomaterial assisted cell therapy, and contributes to advancing the efficacy and design of cell therapies.
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Conferences and Presentations

27/11/17 – The Hatter Cardiovascular Institute Horizon Series 2017: A new era in the management of Type 2 Diabetes- is cardiovascular protection at long last a reality? The Royal College of Physicians, London, UK – Attendance

26/02/18 – 1st London Stem Cell Network Symposium, The Francis Crick Institute, London, UK – Attendance

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10/09/19 – 8th Annual UCL Cardiovascular Symposium and the Launch of the UCL BHF Research Accelerator, London, UK – Attendance

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16/04/21 – Winner of Best Oral Presentation, BHF Annual 4-year PhD Conference, UK
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Abbreviations List

A

ACEI – angiotensin-converting enzyme inhibitors
ARB – angiotensin II receptor blocker
ATP – adenosine triphosphate
αSMA – α-smooth muscle actin

B

BaSO₄ – barium sulphate
BB – beta-blockers
BLI – bioluminescent imaging

C

C – carbon
CO₂ – carbon dioxide
Ca²⁺ – calcium ions
CABG – coronary artery bypass graft
CCR2 – CC-chemokine receptor 2
CHF – chronic heart failure
CM – cardiomyocyte
CSC – cardiac stem cells
CSF-1 – colony stimulating factor-1
CT – computed tomography
cTNT- cardiac troponin T
CVD – cardiovascular diseases

D

DAMPs – damage-associated molecular patterns
DAPI – 4’, 6-diamidino-2-phenylindole
dH₂O – distilled water
DMC – dimethyl carbonate
DNA – deoxyribonucleic acid
DPBS – Dulbecco’s phosphate buffer solution
EB – embryonic bodies
ECG – electrocardiogram
ECM – extracellular matrix
EDX – energy-dispersive X-ray spectroscopy
eGFP – enhanced green fluorescent protein
eGFR – estimated glomerular filtration rate
ESC – embryonic stem cells
ESC-CM – embryonic stem cells-derived cardiomyocytes

FACS – fluorescence-activated cell sorting
FBN – fibronectin
FBS – foetal bovine serum
FMO – fluorescent minus one

GAPDH – glyceraldehyde 3-phosphate dehydrogenase
GFOGER – Gly-Phe-Hyp-Gly-Glu-Arg peptide
GSK3 – glycogen synthase kinase 3

H – hydrogen
H₂O – water
dH₂O – distilled water
HBSS – Hank’s balanced salt solution
HFpEF – heart failure with preserved ejection fraction
HFrEF – heart failure with preserved ejection fraction
HIF-1α – hypoxia inducible factor alpha
HLA – human leukocyte antigens
hPL – human plasma lysate
HU – Hounsfield units
HUVEC – human umbilical vein endothelial cells
H&E – Haemoyalin & Eosin

I

ICG – indocyanine green
IL-10 – interleukin-10
iPSC – induced pluripotent stem cell
iPSC-CM – induced pluripotent stem cells-derived cardiomyocytes

J

K

K+ – potassium ions
KCCQ – Kansas City cardiomyopathy questionnaire

L

LA – left atrium
LAD – left anterior descending
LUC – luciferase
LV – left ventricle
LVEDV – left-ventricular end-diastolic volume
LVEF – left-ventricular ejection fraction
Ly6C – lymphocyte antigen 6C
Ly6G – lymphocyte antigen 6G

M

MHC – major histocompatibility complex
MFI – mean fluorescent intensity
MI – myocardial infarction
MLHFQ – Minnesota living with heart failure questionnaire
M-Mode – motion mode
MMP2 – matrix metallopeptidase 2
MMPs – matrix metalloproteinases
MRA – mineralocorticoid receptor agonist
MSC – mesenchymal stem cells
N
Na+ – sodium ions
NHS – National Health Service
NICE – National Institute for Health and Care Excellence
NIR – near infra-red
NSG – NOD scid gamma
NT-proBNP – N-terminal pro-B-type natriuretic peptide
NYHA – New York heart association

O
O – oxygen

P
PA – pulmonary artery
PBS – phosphate buffered serum
PCL – polycaprolactone
PEG – polyethylene glycol
PFA – paraformaldehyde solution
PGA – poly-glycolic acid
PGS – poly(glycerol sebacate)
PLGA – poly(lactic-co-glycolic acid)
PLA – poly(lactic acid)
PLL A – poly(l-lactic acid)

Q
qPCR – quantitative polymerase chain reaction

R
RA – right atrium
RGD – Arg-Gly-Asp peptide
ROI – region of interest
RT-PCR – reverse transcription polymerase chain reaction
RV – right ventricle
S
SA – surface area
±SD – standard deviation
SEM – scanning electron microscopy
±SEM – standard error of the mean
SFFV – spleen focus-forming virus

T
TC – tissue culture
TESPA – 3-triethoxysilylpropylamine
TGF-β – macrophage-derived transforming growth factor-β
TIPS – thermally induced phase separation
TMP – transmembrane potential
TNF – tumour necrosis factor

U

V
VCAM – vascular cell adhesion molecule 1
VEGF – vascular endothelial growth factor
VTN-N – vitronectin
v/v – volume/volume

W
w/v – weight/volume

Y
YIGSR – Tyr-Ile-Gly-Ser-Arg peptide
Chapter 1: Introduction

1.1 Chronic Heart Failure

Cardiovascular diseases (CVD) are the leading cause of global deaths. CVD are a group of disorders of the heart and coronary circulation, which include ischaemic heart disease. Ischaemic heart disease is a major cause of global morbidity and mortality, accounting for 8.9 million deaths world-wide in 2019\(^1\), where atherosclerosis leading to myocardial infarction (MI) is known to be the major contributor to these cases. MI results from the occlusion of the coronary arteries which provide oxygen-rich blood to the heart. The majority of occlusions are generated from atherosclerotic plaque, composed of fat cholesterol, calcium and other blood components, that hardens over time and narrows the arteries\(^2\). The occlusion in the major coronary artery ruptures and causes the formation of a blood clot at the site of rupture. The clot prevents blood flow and oxygen from reaching the ventricular region of heart\(^3\).

![Figure 1.1: Progression of arterial occlusion by an atherosclerotic plaque leading to myocardial infarction and damage to the muscular wall of the heart. Myocardial infarction (MI). Created with BioRender.](image)


Depending on which major coronary artery is occluded (Figure 1.2), an ischaemic region can develop in the following areas:

(1) **Left main artery**
   Extensive anterior injury

(2) **Circumflex artery**
   Left ventricular lateral injury
   Left ventricular inferior and posterior injury

(3) **Left anterior descending**
   Left ventricular anterior occlusion injury

(4) **Right coronary artery**
   Left ventricular inferior and posterior injury
   Right ventricular injury

**Figure 1.2:** The location of the arterial occlusion affects the location of subsequently developed ischaemic regions in the heart. Created with BioRender.

The occlusion of the coronary arteries produces an ischaemic insult followed by an inflammatory response, via neutrophil and monocyte engagement. The insult results in the death of the cardiomyocytes (CM) which make up the myocardium. The size of the injury is variable and dependent on the nature of the coronary occlusion, but on average 25% of the 2-4 billion resident CM are lost\(^4,5\).

In addition, cardiomyocytes have extremely low cellular replication rate and the heart cannot regenerate the lost myocardium\(^6\). The loss of cardiomyocytes leads to adverse left ventricular remodelling, where surviving cardiomyocytes elongate in response to high blood pressure in order to increase blood input\(^7\). The myocardium becomes thinner and pumps blood less efficiently, which further weakens the heart\(^7\). Additionally, the heart produces non-contractile fibrotic tissue to replace the lost cardiomyocytes which contribute to the irreversible partial loss of ventricular pumping function. The weakened heart and impaired heart contractions eventually results in chronic heart failure (CHF)\(^7\).
1.1.1 Epidemiology
CVD are responsible for the highest number of deaths globally. In 2019, CVD accounted for 32% of the global deaths, with a total of 17.9 million deceased\(^8\). 85% of CVD related deaths are cause by MI and strokes, attributed to a blockage of blood supply to the heart or brain\(^8\). Specifically, ischemic heart disease is the number one cause of death globally, responsible for 8.9 million deaths, and followed by stroke in second place, responsible for 6.2 million deaths in 2019\(^1\). The majority of CVD related deaths (57%) can be attributed to eight lifestyle risk factors: hypertension, hyperglycemia, low rates of physical activity, obesity, hypercholesterolemia and poor diet\(^9\). CVD have a higher prevalence in low and middle income countries, where patients do not have facilitated access to integrated primary health care programs for the early detection and treatment of risk factors\(^10\). This results in the late detection of the disease, after it causes irreversible detrimental changes to the patients' health.

Acute myocardial infarctions have a 30% mortality rate, and the prognosis are variable, dependent on the size of the infarct, the residual left ventricular function and whether the heart was revascularized. An adverse prognosis after MI leads to CHF, which contributes to late morbidity and mortality\(^11\).

In the United Kingdom there are approximately 900,000 people affected by CHF, of which 70% of them result from MI, and this number is expected to increase over the coming years\(^7\). Heart failure results in a burden to a patient’s life due to the deterioration in quality of life, including dietary, travel and exercise restrictions and premature mortality\(^12,13\). CHF also presents an economic burden to the health system. The National Health Service (NHS) spends 1 to 2% (approximately £625 million) of its annual budget towards treatment and care of this condition\(^7\).

1.1.2 Healthy heart anatomy and function
The heart is the organ that pumps blood around the body through the circulatory system, delivering oxygenated blood to the tissues and removing de-oxygenated blood.
The heart is made up of three layers of tissue (Figure 1.3):

1) Pericardium – the thin protective outer layer
2) Myocardium – the thick muscular middle layer that contracts and pumps the blood around the heart
3) Endocardium – the thin inner layer, lining the inside of the heart chambers and surface of the valves.

![Diagram of heart layers](image)

**Figure 1.3: The heart is composed of three tissue layers.** Created with BioRender.

Structurally, the heart is composed of 4 chambers (Figure 1.4), the top two chambers are the right atrium (RA) and the left atrium (LA), and the lower chambers are the right ventricle (RV) and left ventricle (LV). The chambers are separated in the midline by a muscular septum. The unidirectional flow of blood flow through the chambers is modulated by the opening and closing of 4 valves:

1) Tricuspid valve
2) Pulmonary valve
3) Mitral Valve
4) Aortic valve

De-oxygenated blood from the systemic circulation returns to the heart via the RA and the tricuspid valve enables circulation flow into the RV. The RV pumps the blood through the pulmonary valve and into the pulmonary artery (PA). The PA brings de-oxygenated to the lungs, where it becomes oxygenated. Oxygenated blood flows
back from the lungs into the heart via the LA. From the LA, the blood passes through the mitral valve and into the LV. Under high pressure, blood is pumped into the aorta, which returns oxygenated blood into the systemic circulation.

Figure 1.4: Normal heart structure and blood flow. Right atrium (RA), right ventricle (RV), left atrium (LA), left ventricle (LV). Created with BioRender.

The pumping of the heart is regulated by the sinus node. The sinus node is a cluster of cells, located in the superior region of the wall of the RA. The pacemaker automatically produces electrical activity in a cyclical manner, and this initiates every heartbeat. The cluster of specialized cells sends electrical impulses throughout the heart and coordinates the heart’s contractile patterns. These contractile patterns enable the pumping of blood through the respective chambers of the heart and the systemic circulation of de-oxygenated and oxygenated blood.

The electrical simulations are called action potentials, and they are created by the flow of ion fluxes through the membrane of CM. The action potential in CM is composed of 5 phases:
Figure 1.5: The phases of the action potential in cardiac muscle. Transmembrane potential (TMP), sodium ions (Na\(^+\)), calcium ions (Ca\(^{2+}\)), potassium ion (K\(^+\)). Created with BioRender.

The cardiac cycle begins with the depolarization of the sinus node (Figure 1.6), which propagates the action potential to the atria and produces atrial systole. The action potential gets briefly delayed at the atrioventricular node by regions of slow conductance and this enable the contracting atria to fill the ventricles. Upon emptying of the atria, the Tricuspid and Mitral valves close. Following valve closure, the atria begin re-filling, and the electrical impulse moves through the atrioventricular node and Bundle of His, into the bundle branches and the Purkinje fibers. Propagation of the action potential through the Purkinje fibers contract the ventricles. During ventricular depolarization, the sinus node recharges to produce another stimulus, and this is called ventricular repolarization. The electrical activity restarts at the sinus
node and the atrioventricular node repolarizes during ventricular filling. The electrical impulses are generated in a cyclical manner and produce a single heartbeat.

![Cardiac Electrical Conduction System](image)

**Figure 1.6: The cardiac electrical conduction system.** Created with BioRender.

1.1.2.1 Classification

In CHF, the structure and function of the heart become dysfunctional. According to the levels of circulating natriuretic peptides, ejection fraction, structural and diastolic (filling phase of the heartbeat cycle) dysfunction, CHF can be classified into specific sub-types that require different treatments.

1.1.2.2 Left-ventricular Heart Failure

The left ventricle of the heart has thick muscular walls and it provides the most contractile power to pump blood in the circulation. In left-ventricular failure, the myocardium must work harder to compensate for non-contractile scar tissue and maintain sufficient blood supply. Left-ventricular failure can be classified into two subtypes: systolic and diastolic dysfunction (Figure 1.7).
Heart failure with reduced ejection fraction (HFrEF), refers to systolic dysfunction. The LV loses its ability to contract and as a result pumps too little blood in the circulation\textsuperscript{15}.

Heart failure with preserved ejection fraction (HFpEF), refers to diastolic dysfunction. The LV becomes stiff and loses its ability to relax. This results in reduced blood filling in the chambers of the heart and too little blood is pumped from the poorly filled ventricles\textsuperscript{15}.

\textbf{Figure 1.7: Classification of left-ventricular dysfunction in CHF.} Created with BioRender.
1.1.2.3 Right-ventricular Heart Failure

Heart failure can also affect the right side of the heart, which normally collects deoxygenated blood from the circulation and pumps it into the lungs to be replenished with oxygen. RV failure normally occurs secondary to left-sided failure\textsuperscript{16}. Failure of the LV increases the fluid pressure which is transferred back through the lungs and damages the RV\textsuperscript{16}. The damaged RV loses its ability to contract and pump enough blood through the pulmonary circulation to achieve adequate LV filling\textsuperscript{16}. As a result backward failure, where blood backs up in the systemic veins delivering de-oxygenated blood to the heart, causes systemic congestion\textsuperscript{16}.

1.1.2.4 Congestive Heart Failure

Congestive heart failure, refers to a sub-type of heart failure characterized by fluid back up into the lungs and tissues\textsuperscript{15}. Specifically, as the blood flow pumped out of the heart slows down, the return of blood to the heart backs up into the venous return and causes congestion in tissues\textsuperscript{15}. Fluid collection in the lungs can interfere with breathing and ultimately develop in pulmonary edema\textsuperscript{15}.

1.1.3 Diagnosis and therapy for chronic heart failure

Classification of sub-types of CHF is essential to determine the appropriate monitoring and treatment strategy, to improve prognosis.

Diagnosis of CHF in the UK is determined by a combination of blood tests, imaging methods, functional tests and patient history records, as determined by the National Institute for Health and Care Excellence (NICE) guidelines- NG106\textsuperscript{14}. A summary of NG106 guidelines for CHF diagnosis is depicted in Figure 1.8.

Generally, a clinical examination with a detailed patient history will firstly confirm the possibility of heart failure. Next, measurements of N-terminal pro-B-type natriuretic peptide (NT-proBNP) will be taken. High levels of NT-proBNP are associated with a poor prognosis, therefore if patients have NT-proBNP levels above 400ng/l they will be referred to specialist assessment and transthoracic echocardiography\textsuperscript{14}. Transthoracic echocardiography determines the presence of valve diseases, assesses systolic and diastolic function and detects intra-cardiac shunts\textsuperscript{14}. If poor images are obtained, the technique can be compensated by other imaging modalities including cardiac MRI and radionuclide angiography\textsuperscript{14}.
In combination with the NT-proBNP measurements, electrocardiograms are performed and/or other tests (including chest x-rays, blood tests, urinalysis and spirometry) to identify aggregating factors and/or alternative diagnoses.

Figure 1.8: Diagnosing CHF using the NICE NG106 guidelines. Reproduced from the National Institute of Health and Care Excellence. N-terminal pro-B-type natriuretic peptide (NT-proBNP).

Once CHF and the sub-types are identified, specialist assessment can determine the disease monitoring and management strategy. NG106 guidelines to the management of CHF are visually summarized in Figure 1.9. For managing all types of CHF, a general pharmacological treatment regime is recommended. The regime includes a standard prescription of diuretics for the relief of congestive symptoms and fluid retention. Lifestyle advice is also provided to all CHF patients, particularly on the aspects of diet and salt restrictions, smoking and alcohol management.
Figure 1.9: Managing CHF using the NICE NG106 guidelines. Reproduced from the National Institute of Health and Care Excellence. Angiotensin-converting enzyme inhibitor (ACEI), beta-blocker (BB), mineralocorticoid receptor agonist (MRA), angiotensin II receptor blockers (ARB), estimated glomerular filtration rate (eGFR).
Following general management, a tailored pharmacological treatment for CHF is designed upon the diagnosis of CHF sub-type.

Patients with HFpEF, are generally maintained on diuretics management and treatment for comorbidities, such as the prescription of anti-coagulants if patients have atrial fibrillation\textsuperscript{14}. These patients are offered cardiac rehabilitation programs and managed to retain their preserved ejection fraction to avoid further heart problems.

First-line treatment for patients with HFrEF, includes the prescription of angiotensin-converting enzyme inhibitor (ACEI) and a beta-blocker\textsuperscript{14}. ACEI block the conversion of angiotensin I to angiotensin II, which reduces blood pressure, increases parasympathetic activity in the heart to manage cardiac arrhythmias and reduces myocardium wastage. If ACEI are not tolerated, angiotensin II receptor blockers can be prescribed\textsuperscript{14}. Beta-blockers are beta-adrenoceptor antagonists, and they inhibit epinephrine-induced cardiac stimulation and enable the management of cardiac arrhythmias and elevated blood pressure. If the symptoms persist with first-line treatment, mineralocorticoid receptor antagonists (MRA), are prescribed and the drugs antagonize the action of aldosterone at mineralocorticoid receptors\textsuperscript{14}. Ultimately, if the symptoms of CHF cannot be managed by first-line and second-line treatment, specialised treatment gets recommended to include drugs such as ivabradine and digoxin (Figure 1.9)\textsuperscript{14}.

The initial pharmacological aims are to improve life expectancy and quality of life. Combinative administration of ACEI, diuretics and beta-blockers is used to reduce blood pressure and volume; however, it does not inhibit the progressive severity of the condition. If CHF becomes more severe, where cardiac function and symptoms cannot be controlled by pharmacological treatment alone, interventional therapies are recommended\textsuperscript{14}. Initial interventional therapies, include cardiac resynchronisation therapy and implantable cardioverter defibrillators\textsuperscript{14,17}. Patients receive an implantable cardiac rhythm device to monitor and activate the atria and ventricles independently\textsuperscript{17}.

Pharmacological and interventional therapies have significantly reduced the mortality and slowed the progression of ventricular dysfunction; however, they have cannot address the primary deficit in heart failure. The range of solutions are not able to
rescue the function of damaged cardiomyocytes and fail to manage the progression of the disease. Therefore a heart transplant often becomes the only remaining solution.\textsuperscript{18} A heart transplant is not viable for all the patients that are affected by CHF. This is due to the limited supply of available and compatible organs.

Hence, there is a clear need for the development of a new therapies designed specifically to stop the progression of CHF.

1.1.3.1 New therapies

Development of novel therapeutics for CHF has been slow. For drug discovery, in the last 20 years only 2 new drugs have been approved by the FDA for CHF treatment, valsartan and ivabradine\textsuperscript{19}. NHS guidelines only prescribe these as specialist treatment for a subset of patients\textsuperscript{14}. Interventional therapy also remains compensatory (i.e. dialysis to reduced afterload) or disease modulatory (i.e. suppression of vasoactive peptides and modulation of cardiac rhythm)\textsuperscript{19}.

Novel drugs and therapeutic devices have improved the quality of the patients’ life; however, they have not decreased morbidity and mortality. To address this unmet need, research projects have moved to the development of therapeutics supportive of cardiac regeneration. Novel therapeutics included, cell-derived, gene-derived, cell-independent and tissue engineering approaches. Of interest in this project, was the utilization of cardiac cell therapies.

1.2 Cell therapies

Over the past 20 years, cell-based therapies have been investigated for the regeneration of damaged heart tissue arising from ischaemic insults\textsuperscript{20,21}. Cell-based therapies were originally designed to achieve remuscularization of the myocardium and restoration of function post-MI. Additionally, remuscularization provided the opportunity of reducing irreversible fibrosis because of chronic injury. This chapter reviews the development of cardiac cell therapies to justify the selection of induced pluripotent stem cells for this research project.

The development of cardiac cell therapies began with the utilization of adult cells which further developed through two stages, first- and second- generation cardiac adult-cell therapies. The trend in the development of a clinical product, moved from the delivery of unselected cells to the delivery of lineage specific cells for the target
organ (Figure 1.10). Specifically, delivery of cells from the heart or with a cardiac-specified lineage. The results of recent IHD and CHF cell therapy clinical studies and associated meta-analyses have been previously reviewed by Madeddu.  

1.2.1 Skeletal Myoblasts
The first clinical trials for cardiac cell therapy, delivered intramyocardial cell suspensions of skeletal myoblasts to patients suffering from ischaemic cardiomyopathy and undergoing coronary artery bypass graft (CABG). The trial produced optimistic safety data and this resulted in the establishment of the double blind, randomized MAGIC trial. 97 patients with CHF were randomly assigned to receive either, a low concentrate, a high concentrate of skeletal myoblasts, or placebo. The study did not meet its primary endpoint, of improvement in left-ventricular ejection fraction (LVEF), and raised concerns on the development of arrhythmias associated with a lack of electromechanical coupling of the graft cells to the host’s myocardium. This resulted in a decline of trials utilizing skeletal myoblasts for cardiac cell therapy and an inclined interest in the usage of other cell types.
1.2.2 Bone marrow-derived cells

Bone marrow-derived cells were subsequently investigated for cardiac cell therapy. These cells were selected for practical reasons, associated with the ease of availability, processing and low cost. Scientifically, this was supported by a seminal and controversial paper which showed that transplantation of bone marrow stem cells, positive for surface marker c-kit, could regenerate an infarcted mouse heart\textsuperscript{26}. Those results were never reproduced. However, these findings translated into a clinical trial which showed that administration of autologous mononuclear bone marrow cells into the infarcted artery during coronary angioplasty, significantly reduced infarct size\textsuperscript{27}. The animal studies coupled with the clinical trial, led to an explosion of interest in the use of bone marrow derived cells in MI and CHF therapy that resulted in the publication of large and international clinical trials\textsuperscript{28–30}. The lack of research into the basic science to support the use of bone marrow-derived cells, produced several studies with contradicting results. The efficacy of bone marrow-derived cells at repairing the myocardium was not reproducible\textsuperscript{31–33}. The basis of the discrepancy was unknown and created confusion with the interpretation of clinical trial outcomes, which further lead research into investigating different subsets of bone-marrow derived cells. The suggested repair mechanism of cell therapy switched from cellular trans-differentiation to mechanically support myocardial repair, to a non-contractile mechanism with a direct cellular or paracrine mechanism.
1.2.2.1 Unselected bone marrow-derived cells

Unselected bone marrow-derived cells comprise of progenitor, stromal and hematopoietic cells. The heterogeneous population of cells, have distinctive phenotype and function. The regenerative potential of unselected bone marrow-derived cells was limited and cell populations at different stages of maturity showed the least efficacy\textsuperscript{34}. Hendriksen \textit{et al.} were the first to report that CABG supported with intramyocardial injections of unselected bone marrow-derived cells improved cardiac contractility in regions of nonviable fibrosis, but did not improve LV function\textsuperscript{35}. The negative results were later supported by other investigations\textsuperscript{36,37}.

Revascularization surgery concomitant to stem cell treatment, introduced the possibility of a confounding variable and therefore CABG was later removed in the FOCUS-CCRTN trial\textsuperscript{38}. Patients with CHF received trans-endocardial administration autologous bone marrow-derived cells or placebo. At the 6 months endpoint, the primary outcomes were not met: changes in lung function, heart function and necrosis at the infarct zone were not significantly different from the placebo group\textsuperscript{38}. A negligent outcome was also seen in the TAC-HFT trial, where patients with ischaemic cardiomyopathy and reduced LVEF received trans-endocardial administration autologous bone marrow-derived cells and did not improve in the walking test nor show a decrease in infarct size compared to placebo\textsuperscript{39}. Interestingly, these outcomes improved in patients that received autologous mesenchymal stem cells (MSC)\textsuperscript{39}.

The lack of therapeutic efficacy of unselected bone marrow-derived cells was postulated to be due to the patient-dependent variability of cellular subcultures which produced variable regeneration\textsuperscript{40}. The variability in regenerative potential could have been further limited by the patient’s diseased phenotype\textsuperscript{41}.

1.2.2.2 Purified hematopoietic stem and endothelial progenitor cells

Unpurified bone marrow-marrow derived cells have been purified in attempt to produce CD34 and/or CD133 positive hematopoietic progenitor cells. The application of purified progenitor cells was therapeutically successful in patients with refractory angina and this was consistent with the angiogenic properties of CD34 and CD133 positive cells\textsuperscript{42}. However, the therapeutic efficacy of purified progenitor cells in
patients with heart failure remains unclear. Stamm et al. initially showed that patients in a non-randomized study which received co-administration of purified CD133 positive cells via intramyocardial injections, in combination with CABG surgery, resulted in improved LVEF versus patients that received CABG surgery alone\textsuperscript{43}. However, when the group tested the reproducibility of these results in a more rigorous trial, with randomization, a placebo control and blinded assessment, they could not re-confirm the efficacy of CD133 positive cell treatment compared to the placebo treatment\textsuperscript{44}.

1.2.2.3 Mesenchymal stem cells

Out of all bone marrow-derived cells, MSC have shown the greatest cardiac therapeutic potential in terms of cardiac regeneration and clinical translatability. The practicality of MSC has been shown by their great availability, ease of scalable production, efficiency after cryopreservation and importantly their strong paracrine effects (angiogenic, anti-inflammatory and immunoregulatory). The earliest use of MSCs was supported by non-cardiac applications before application to CHF. Majority of investigated MSC therapy trials, utilize bone-marrow derived MSC and a minority utilize umbilical cord- or adipose tissue- derived cells. Despite the explosion in the number of MSC clinical trials, the outcomes can be difficult to interpret to the variation in MSC population identity, phenotype, and dosage. Menasché categorized the populations of MSC applied to CHF clinical trials as unmodified, engineered, enriched and antigenically selected\textsuperscript{34}. The specifications and outcomes of the main MSC clinical trials for CHF have been summarized in Table 1.1.
<table>
<thead>
<tr>
<th>Trial</th>
<th>Design</th>
<th>Patient cohort</th>
<th>Treatment specifications</th>
<th>Primary outcome</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unmodified MSC</strong></td>
<td>- Randomized</td>
<td>- Ischaemic HF</td>
<td>- Intramyocardial injection</td>
<td>Change in left ventricular end-diastolic volume (LVEDV)</td>
<td>- Improved LVEDV and LVEF in a dose-dependent manner</td>
</tr>
<tr>
<td>MSC-HF</td>
<td>- Double blind</td>
<td>- LVEF&lt;45%</td>
<td>- 77.5±67.9 x10^6 cells</td>
<td></td>
<td>- No changes in surrogate markers, including functional indices, 6-minute walking test and quality of life questionnaire compared to placebo control</td>
</tr>
<tr>
<td></td>
<td>- Phase I/II</td>
<td>- n=60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHART-146</td>
<td>- Prospective</td>
<td>- Symptomatic</td>
<td>- Intramyocardial injection</td>
<td>- Categorized all-cause cell death</td>
<td>- No significant difference in treatment groups on:</td>
</tr>
<tr>
<td></td>
<td>- Randomized</td>
<td>- Ischaemic HF</td>
<td>- Changes in heart failure events</td>
<td></td>
<td>o All-cause cell death</td>
</tr>
<tr>
<td></td>
<td>- Double blind</td>
<td>- LVEF&lt;35%</td>
<td>- MLHFQ score</td>
<td></td>
<td>o Heart failure events</td>
</tr>
<tr>
<td></td>
<td>- Placebo control</td>
<td>- n=271</td>
<td>- 6-minute walking test</td>
<td></td>
<td>o MI</td>
</tr>
<tr>
<td></td>
<td>- Phase III</td>
<td>-</td>
<td>- LVEDV</td>
<td></td>
<td>o Stroke</td>
</tr>
<tr>
<td>Butler et al.47</td>
<td>- Randomized</td>
<td>- Non-ischaemic</td>
<td>- All-cause mortality</td>
<td>- Treatment did not significantly impact LVEF</td>
<td>- Significant benefit to the stratified group of patients with largely dilated ventricles which received less than 19 injections</td>
</tr>
<tr>
<td></td>
<td>- Single blind</td>
<td>cardiomyopathy</td>
<td>- All-cause hospitalization</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Placebo control</td>
<td>- LVEF&lt;40%</td>
<td>- Adverse events</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cross over</td>
<td>- n=22</td>
<td>- LVEF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Phase Ila</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enriched MSC</strong></td>
<td>- Randomized</td>
<td>- Heart failure</td>
<td>- Trans endocardial injection</td>
<td>- All-cause mortality</td>
<td>- Protocol adhering patients in the treatment group showed significant reduction in the number of detrimental cardiac events compared to the placebo group</td>
</tr>
<tr>
<td>ixCELL-DCM</td>
<td>- Double blind</td>
<td>- LVEF&lt;35%</td>
<td>- MSC population enriched with CD90+ MSC and CD14+ macrophages</td>
<td>- Cardiovascular-related hospitalization</td>
<td>- No changes between the treatment and placebo groups seen in:</td>
</tr>
<tr>
<td></td>
<td>- Placebo control</td>
<td>- n=109</td>
<td>- 12-17 dosages of 0.4ml Ixmyelocel T</td>
<td>- Unplanned clinical visits for the treatment of decompensated HF</td>
<td>o LVEF</td>
</tr>
<tr>
<td></td>
<td>- Phase Ila</td>
<td>-</td>
<td></td>
<td></td>
<td>o LVEDV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>o NYHA class</td>
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<tr>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td>o 6-minute walking test</td>
</tr>
<tr>
<td><strong>Antigenically selected MSC</strong></td>
<td>- Randomized</td>
<td>- CHF</td>
<td>- Trans endocardial injection</td>
<td>- Safety</td>
<td>- Significant reduction in major detrimental cardiac events (HF hospitalization, cardiac</td>
</tr>
<tr>
<td>Perin et al.49</td>
<td>- Phase II</td>
<td>- LVEF&lt;40%</td>
<td>- Safety</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Participants</td>
<td>Intervention</td>
<td>Outcome</td>
<td>Status</td>
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</tr>
<tr>
<td>DREAM-HF</td>
<td>Randomized - Double blind - Placebo control - Phase III</td>
<td>n=60</td>
<td>Selected STRO1+ and STRO3+ allogeneic MSC - 25×10^6, 75×10^6 or 150×10^6 cells</td>
<td>Occurrence of major detrimental cardiac events</td>
<td>death, or resuscitation from cardiac death) in the highest dose group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n=600</td>
<td>Trans endocardial injection - Selected STRO3+ allogeneic MSC</td>
<td>Time to non-adverse recurring first decompensated HF event in the presence of major detrimental cardiac events</td>
<td>On going</td>
</tr>
</tbody>
</table>

Table 1.1: Overview of the main MSC clinical trials for CHF. Adapted from Menasché. Mesenchymal stem cells (MSC), heart failure (HF), left-ventricular ejection fraction (LVEF), left-ventricular end-diastolic volume (LVEDV), myocardial infarction (MI), Minnesota living with heart failure questionnaire (MLHFQ), Kansas City cardiomyopathy questionnaire (KCCQ), New York heart association (NYHA).
1.2.2.4 Meta-analysis of bone marrow-derived cell therapies

Individual studies for the application of respective cell therapies suggested efficacy, however those initial trials were small, without randomization, cohesive enrollment criteria or primary endpoints. The latest trials with larger patient cohorts and improved study design have struggled to show improved outcomes over the standard medical therapies, in addition to producing conflicting results. To provide a comprehensive insight into the impact of bone marrow-derived cell therapies, the Cochrane group performed a meta-analysis on the randomized controlled trials that were published up to 2016\textsuperscript{50}. The review, evaluated the clinical evidence on the safety and efficacy of of bone marrow-derived cell therapies for chronic ischaemic heart disease and congestive HF. The review included 38 randomized trials, involving 1907 participants, 15 studies included patients with chronic heart failure, 17 with congestive heart failure and 7 with refractory angina, which are different clinical settings\textsuperscript{50}. Overall, the review found that the evidence for the efficacy of bone marrow-derived cell therapy is of low quality, lacking evidence for efficacy in improved LVEF measured by MRI, composite mortality, non-fatal MI and/or HF re-hospitalization\textsuperscript{50}. Cell therapy was associated with a lowered incidence of non-fatal MI and arrhythmias, however these results were confounded by the low event rates which leads to a lack of precision\textsuperscript{50}. Interestingly, the most consistent finding in the review was the overall safety of cell therapy as only a small number of procedure-associated adverse events were recorded\textsuperscript{50}.

While the conflicting reports that contributed to this Cochrane meta-analysis were being published, stem cell researchers were becoming aware that the regenerative potential of bone marrow-derived cells might not be realized. This led to the development of a second-generation cardiac cell therapy, the utilization of cardiac-specific cells.
1.2.3 Cardiac-specific cells
1.2.3.1 Cardiac stem cells

Basic science studies by Anversa’s group proposed that the heart contains stem and/or progenitor cells, expressing the same c-kit marker that they used in the identification of regenerative bone marrow derive cells\textsuperscript{51}. The studies suggested that the reparative capacities of these stem cells could be transformed into a more effective therapeutic, by using the most appropriate stem cell naturally suited to repair their respective resident tissue. These cells were identified by immunostaining and transplantation, but without demonstration of stemness by lineage tracing. Beltrami \textit{et al.} suggested that the cardiac stem cells (CSC) are self-renewing and enable homeostatic large turn-over of cardiomyocytes in rodents and humans\textsuperscript{51}. \textit{In vivo} studies in infarcted dog and rat models further suggested that CSC can be identified, isolate and transplanted with ease and resulted in the improvement of cardiac function\textsuperscript{52,53}. These findings produced collaborations that reported positive heart regeneration by administration of c-kit+ cells\textsuperscript{54,55} and lead to the isolation of more presumed CSC\textsuperscript{56}. Investigations into CSC, led to the development of 3D CSC culture, characterised by cells outgrowing \textit{in vitro} from endomyocardial biopsies of the RV\textsuperscript{57}. These were called cardiospheres, which showed \textit{in vitro} differentiation into cardiomyocytes and produced an improvement of cardiac function after administration to a murine model of MI\textsuperscript{57}.

Kit+ CSC entered the clinic in the SCiPIO trial, the first randomized, place controlled study where patients with ischaemic cardiomyopathy and LVEF<40% received autologous cell therapy, derived from atrial biopsies, during surgical revascularization at 4 months post-MI\textsuperscript{58}. The study reported good safety data and improvements in cardiac function, at 1 and 2 years post-administration\textsuperscript{58}. However the study was initially flagged with integrity issues and warnings against the data analysis\textsuperscript{59}. To add to the controversy, literature reported that Kit+ cells are more likely to produce vascular cells than CM\textsuperscript{60} and that Kit+ CSC rapidly senesce in culture\textsuperscript{61}.

Cardiospheres also entered the clinic in the CADUCEUS trial\textsuperscript{62}. Autologous cardiospheres were administered to patients, who experienced recent MI, via
intracoronary infusion. At 6 months, the trial showed that cardiosphere administration was safe but compared to the standard of care, treatment did not benefit global cardiac function. Ultimately, the theory of cardiac stem cells was eliminated as investigations failed to corroborate the stemness of cardiac kit+ cells. Pouly et al. reported that c-kit+ cells biopsied from HF patients, were actually mast cells. Multi-isotope mass spectroscopy demonstrated that the murine heart cardiac replication rate is very low, about 1% a year and this can be fully accounted by mitosis of resident CM. Van Berlo et al. genetically lineage traced the faith of cardiac Kit+ cells using Cre-recombinase technology in a murine model. The study showed that during normal aging or injury through MI, the level of new CM formation from cardiac Kit+ cells is below 1/1000. Additional investigations produced conflicting results which have been reviewed in detail by Chien et al. and resulted in reports to the NIH board of research integrity. Ultimately the Anversa’s group was found guilty of scientific misconduct which led to the retraction of numerous papers, including retraction of the SCIPIO study in 2019 and halted enrolment to the CONCERT-HF due to concerns on the scientific foundations of the trial.

1.2.3.2 Pericytes

Pericytes have been isolated from several tissue sources and have been extensively studied for the treatment of heart disease. Paolo Madeddu’s group reported the isolation of saphenous vein-derived pericytes from small tissue biopsies or left-overs of cardiac surgery. The isolation method generated clinical grade cells with proangiogenic and clonogenic potential. Transplantation of saphenous vein-derived pericytes produced improvements in cardiac function in murine models of non-reperfused MI and synergistic activity was observed when transplanted in combination with cardiac stromal cells. However, the therapeutic effects of pericyte transplantation were less effective in a swine model of reperfused MI. Pericyte therapy showed pro-angiogenic and antifibrotic benefits, however failed to improve left ventricular function.

Cardiac pericytes have been proposed as a tissue specific solution for the treatment of heart disease. Cardiac pericytes are isolated from the perivascular region
around blood vessels in the atrial and ventricular regions of the human myocardium. Cardiac pericytes have shown to maintain microvascular function when cultured \textit{in vitro} and transplanted \textit{in vivo}. However, the invasive nature of acquiring cardiac pericytes through a cardiac biopsy presents a clinical limitation to this cell therapy. To overcome this limitation, strategies to reprogram endogenous pericytes by repurposing clinically available drugs have been proposed.

1.2.4 First-generation adult cell products and the paracrine hypothesis

To date, despite some studies showing limited beneficial effects of cell therapy (measured by improvement of ventricular function), clinical trial results have generally been disappointing, with studies not meeting their primary outcome measures and producing minimal improvement in cardiac function. Several reasons have been suggested to account for the poor outcome observed. These include the limited regenerative potential of unspecific stem cells and the paracrine hypothesis associated to poor cellular engraftment.

The paracrine hypothesis suggests that the transplanted cells do not engraft but secrete soluble factors that encourage cardiac repair through mechanisms of angiogenesis, protection of native cells and attenuation of inflammation. This hypothesis has been supported by studies that demonstrate that delivery of hypoxic conditioned medium from bone marrow-derived mesenchymal stem cells overexpressing Akt has similar beneficial effects to intramyocardial injection of Akt-expressing mesenchymal stem cell therapy.

Hence, it seems widely accepted that the first-generation of adult cell products seem to produce a regenerative environment via paracrine factors and/or angiogenesis, whereas to provide an unlimited source of cardiomyocytes, pluripotent stem cells are being investigated.
1.2.5 Pluripotent stem cells
In contrast to adult cell products, pluripotent stem cells can divide indefinitely and produce any type of human cell, including unambiguous CM. Given the potential of these renewable differentiated CM to re-muscularise the infarcted heart, there has been high and increasing interest in the use of pluripotent stem cells, The second-generation cell therapy includes embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC).

1.2.5.1 Embryonic stem cells
ESC are isolated from the inner cell mass of the blastocyst. Exposure of ESC to specific chemical cues in vitro can differentiate ESC into any somatic cell type. In the early research stages for the application of ESCs to cardiac regeneration, it was believed that the cardiac milieu would provide the critical cell-to-cell cues or chemical cues to differentiate ESC into a cardiac phenotype and enable integration to the host tissue. However, administration of ESC suspensions into murine hearts resulted in the formation of teratomas and produced an immunogenic reaction which resulted in graft rejection at 4 week post-administration.\(^{78,79}\)

By differentiating ESC into CM in vitro and prior to administration, the teratogenic potential of ESC pluripotency was eliminated. Human ESC-derived CM (ESC-CM) were injected in the left ventricular wall of healthy athymic murine hearts and after 4 weeks, the graft consisted predominantly of CM, teratoma formation did not occur and the implant exhibited substantial angiogenesis.\(^{80}\) Human ESC-CM grafts were shown to integrate in vitro to mature murine CM via the formation of structural and electromechanical connections.\(^{81}\) The same human ESC-CM cell grafts were transplanted into swine hearts with complete atrioventricular block.\(^{81}\) The grafted cells were shown to integrate to the blocked heart and electrophysiological mapping showed pacing of the swine heart via the graft’s ectopic rhythm.\(^{81}\)

Transplantation of ESC-CM into murine models of MI, produced a reproducible and long-term improvement in LVEF, coupled with electrical integration to the host myocardium.\(^{82,83}\) The safety of the electrophysiological behaviour of grafted ESC-CM in small animal models was shown by Shiba et al. by implantation of ESC-CM grafts in a guinea pig cardiac injury model.\(^{84}\) ESC-CM grafts expressing a fluorescent
calcium sensor GCaMP3 and a pro-survival cocktail were administered through intracardiac injections into injured guinea pig hearts\textsuperscript{84}. 28 days post-administration, functional improvements to the injured areas were seen, along with 1:1 host graft coupling which was measured by GCaMP3 fluorescent signalling correlated with host electrocardiogram (ECG)\textsuperscript{84}. Graft-treatment also reduced spontaneous- and induced- ventricular tachycardia, suggesting that ESC-CM grafts in injured hearts contract synchronously with the host tissue and can protect against arrhythmias\textsuperscript{84}.

Pre-clinical large animal studies have shown that clinical-scale ESC-CM transplantation is feasible. Chong \textit{et al.} produced a clinical quantity (1 billion per batch which is the approximate number of cell loss during human MI) of ESC-CM which were cryopreserved with good viability\textsuperscript{85}. A non-human primate model (macaque) of MI followed by reperfusion, received 1 billion cryopreserved ESC-CM via intra-myocardial injection, resulting in significant remuscularization\textsuperscript{85}. 2 weeks after implantation, the grafts were perfused by the host vasculature and electromechanical junctions were visible between graft and host CM\textsuperscript{85}. The grafts showed regular calcium transients that were coupled to the host ECG, however the ESC-CM showed progressive but incomplete maturation up to 3 months\textsuperscript{85}. Arrhythmias were observed in a subset of animals after receiving ESC-CM graft treatment, which had not been seen in previous small animal models\textsuperscript{85}. Electrophysiology indicated that arrhythmias arise from the ectopic pacemaker activity from engrafted cells rather than delayed re-entry arrhythmias from slow conductance loops\textsuperscript{86}. Interestingly, the arrhythmias were transient as they lasted 2-3 weeks before the heart returned to normal rhythm\textsuperscript{86}. ESC-CM graft intolerance was also seen in another large animal model consisting of a 20-30kg infarcted swine model\textsuperscript{87}. Graft transplantation improved cardiac function, but resulted in the occurrence of severe arrhythmias\textsuperscript{87}. The arrhythmias were more severe than the arrhythmias observed from the primate model, but similarly they self-resolved within 1 month of graft transplantation\textsuperscript{19,86,87}.

Graft-induced arrhythmias were not detected in smaller animal models, but they were seen in a reproducible manner in larger animal models. It is believed that the difference in arrhythmia occurrence is related to variation in the host heart size and
rate\textsuperscript{88}. The accepted hypothesis is that the arrhythmias arise from graft immaturity, the arrhythmias self-resolve once the graft is electrically matured and drops the pace making rates below the rate of the host’s sinus node\textsuperscript{19}. This phenomenon may be accentuated in larger animals that receive larger cell grafts\textsuperscript{88}. Additionally, it has been proposed that faster heart rates in smaller animals favour native conduction pathways over graft automaticity\textsuperscript{88}. While the slower heart rates in larger animals, increases the susceptibility to graft automaticity and ventricular arrhythmias\textsuperscript{88}.

Despite the occurrence of arrhythmias, the main barrier to ESC-CM therapy are the ethical controversies associated with their derivation from early embryos. To address this challenge, investigators are now turning to other potentials sources of cells and the discovery of induced pluripotent stem cells (iPSC) has redefined the potential of regenerative medicine.

1.2.5.2 Induced pluripotent stem cell

iPSC are somatic stem cells reprogrammed into pluripotent stem cells using a defined reprogramming protocol. Yamanaka’s group pioneered the technology in 2006 after showing that it is possible to convert mouse fibroblasts into embryonic stem cell-like colonies by the introduction of four gene-encoding transcription factors: Oct 3/4, Sox2, Klf and c-Myc (Figure 1.11)\textsuperscript{89}. 


Figure 1.11: Somatic reprogramming of adult cells using Yamanaka’s iPS reprogramming factors to produce iPSC. Created with BioRender.

A year later, Yamanaka’s group reported the production of human iPSC with embryonic stem-cell like properties from human fibroblast cultures using the same defined reprogramming factors. To date, iPSC have been derived from a variety of adult tissues, including keratinocytes from hair, blood cells and exfoliated renal tubular epithelial cells obtained from urine. iPSC can be easily obtained from a large pool of sources and bypass the ethical controversies of manipulating embryonic tissues. iPSC can propagate indefinitely and can be guided into specific lineages, providing a single inexhaustible source of cells that could replace damaged or diseased tissue (Figure 1.11), including the human myocardium.

Toivonen et al. compared multiple ESC and iPSC lines for their capacity to differentiate into CM and other lineages and reported variations in differentiation potential and transgene persistence. Further work by Sepac et al. determined that the differences in cardiomyogenic potential was cell line dependent. Nonetheless, differentiated ESC- and iPSC-derived CM (iPSC-CM) demonstrate significantly identical phenotypes in terms of efficiency in forming contracting CM, CM contraction...
rates, upregulation of cardiac genes and concomitant downregulation of pluripotency genes, proliferation, sarcomere organization, electrical activity and pharmacological modulation\textsuperscript{97,98}.

\textit{In vivo} investigations indicate the potential of iPSC-CM for cardiac regeneration, in a similar manner to the efficacy of ESC-CM therapy. Funakoshi \textit{et al.} compared the engraftment efficiency of iPSC-CM grafts of different cardiac maturity, via bioluminescence tracking\textsuperscript{99}. Intra-myocardial injections of undifferentiated iPSC, day 4 mesodermal cells, day 8, day 20 and day 30 iPSC-CM suspension delivered into immune compromised murine hearts\textsuperscript{99}. 20 days old iPSC-CM showed superior efficacy in terms of cellular engraftment and cardiac functional improvement as measured by ECG\textsuperscript{99}. The graft reached maximum proliferative growth 3 months post-administration, after which the graft started progressively maturing up to 6 months\textsuperscript{99}.

The long term improvement in cardiac function and cell maturation of large ESC-CM grafts transplanted into the infarcted hearts of larger animal models, was also seen in an allogeneic primate model that received rhesus monkey iPSC-CM\textsuperscript{100}. Major histocompatibility complex (MHC) I and II play a critical role in the immune response post-transplantation\textsuperscript{101} and graft vs. host disease\textsuperscript{102}. Immune rejection can occur from the innate immune response in the acute phase of transplantation and the adaptive immune response which develops more latently through the detection of external antigens presented by MHC. MHC-matching transplants are a potential approach to evade allogeneic graft rejection. Shiba \textit{et al.} identified a MHC-class I and II homozygous animal as an iPSC donor, from which they isolated skin fibroblast and established an iPSC cell line\textsuperscript{100}. The cell line was transfected to develop a fluorescent calcium indicator to show that transplanted cells engraft and couple with the host CM\textsuperscript{100}. The MHC-matched iPSC were differentiated and enriched to cardiac troponin positive CM, via glucose starvation, and heat shocked before being cryopreservation\textsuperscript{100}. 400 million iPSC-CM were transplanted into MHC-matched immunosuppressed primates and received follow up after 12 weeks\textsuperscript{100}. Cardiac contractile function improved after 1 month of transplantation and the improvement was sustained at 3 months, compared to cell-free vehicle control\textsuperscript{100}. The graft reduced the infarct zone and electrically coupled to the host CM\textsuperscript{100}. However,
similarly to the ESC-CM treatment, the incidence of ventricular arrhythmias was transient but significantly increased compared to the vehicle controls\textsuperscript{100}. Of importance, the investigation suggested that allogeneic transplantation was sufficient to regenerated the infarcted myocardium without rejection up to 3 months, using a commonly used immunosuppression regimen\textsuperscript{100}. Specifically, long-term allogeneic iPSC-CM allografts transplantation can be supported without rejection, without the requirements of high levels of immunosuppression.

iPSC can be used in an autologous manner and provide patient-matched treatment without the risk of immune rejection. However, the production and differentiation of patient-specific cell lines is a lengthy and costly process. Generally the cost of generating and validating a research grade iPSC line is $10,000-25,000 requiring 6-9 months, starting from patient recruitment, and requiring further 3-6 months to upscale the production of cellular derivatives\textsuperscript{103}. The cost of producing clinical grade iPSC lines is higher, estimated at $800,000\textsuperscript{103}. Because of these limitations both industry and academia, are moving towards the concept of allogeneic cell products and therapies.

A current approach is the collection of iPSC generated from patients with homozygous MHC loci, to generate exponentially higher MHC matches with the general population. Libraries of allogeneic super-donor cells that Human Leukocyte Antigens (HLA)-match 80\% of Japan’s patient’s populations are being established by CiRa research centre to enhance immunological compatibility for future treatments\textsuperscript{104,105}. In vivo studies have shown that MHC-matched iPSC implantation in an allogeneic rat host completely evaded immune rejection\textsuperscript{106}. Further differentiation and implantation of MHC-matched iPSC-derived neural precursors into allogeneic minipig recipients was also be achieved using temporary immunosuppression\textsuperscript{107}. These studies have been promising, suggesting that these therapies can be engineered for universal transplantation and have the potential to become a more cost- and process-effective off the shelf product\textsuperscript{108}. However, to date immunosuppression is still required to prevent rejection, as shown by the reduced immunogenicity of iPSC-CM that Shiba et al. transplanted into MHC-matched immunosuppressed primates\textsuperscript{100}. Similarly, Badin et al. showed that grafting of MHC-matched iPSC-derived neurons into the lesion brains of macaque monkeys required
reduced immunosuppression, as MHC-matching to evade rejection was insufficient\textsuperscript{109}.

The application of stem cell-derived CM is backed by decades of basic science research and proof-of concept preclinical studies that show promising regenerative results all the way from in vitro models to more clinically relevant large animal models. Prior attempts at regenerating the infarcted heart with non-specific and undifferentiated cells therapies did not meaningfully retain the engrafted cells and therefore suggest that any resulting benefit was elicited from paracrine activity. In contrast, iPSC-CM transplantation produced larger remuscularization with contractile and electrochemically coupled grafts, which suggests a direct functional benefit from robust engraftment. Despite these promising results, conclusive evidence is required to isolate the therapeutics benefits of contractile and non-contractile effects of cell therapy. This could pave the way to maximising the efficacy of cell therapy and solving the remaining safety concerns.

1.2.6 Limitations of cell therapies

The preclinical benefits of cardiac cell therapy have only produced modest improvements in clinical trials, irrespective of the cell type used. These observations may be explained by untargeted delivery routes and poor engraftment of the cell therapy\textsuperscript{110}. To ameliorate or treat CHF, the delivered graft tissue needs to replace a substantial fraction of the injured myocardium that was lost to infarction. The therapeutic effect of cell therapy is premised on cell survival. It is important that transplanted cells are retained in the heart for long enough to engraft and send paracrine signals to the host cells for repair and regeneration. Therefore, maximal cell retention is essential for the success of cell therapies for cardiac regeneration. Studies utilizing skeletal myoblasts indicated that graft size correlates with direct functional improvement\textsuperscript{111,112}.

The cell retention and outcomes of cell therapy products to the heart have been thoroughly reviewed by Li et al.\textsuperscript{110} and summarised in Table 1.2. Regardless of cell type and delivery routes, clinical and pre-clinical studies have reported low cell retention in the heart.
<table>
<thead>
<tr>
<th>Delivery Route</th>
<th>Species and Setting</th>
<th>Transplanted Cell Type</th>
<th>Cell Retention Rate, %</th>
<th>Time Point</th>
<th>Quantification Method</th>
<th>Functional Recovery</th>
<th>Reference</th>
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<td>Intra coronary</td>
<td>Pig MI model</td>
<td>Bone marrow mononuclear cells</td>
<td>1.0±0.8</td>
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<td>Pig MI model</td>
<td>Cardiac-derived progenitor cells</td>
<td>18</td>
<td>4 h</td>
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<td>↑ LVEF</td>
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<td>Mesenchymal stem cells</td>
<td>13.74</td>
<td>4 h</td>
<td>Radiolabeling+PET-CT</td>
<td>No data available</td>
<td>Gathier et al; 2019</td>
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<tr>
<td></td>
<td>Pig MI model</td>
<td>Mesenchymal stem cells</td>
<td>1.7±0.1</td>
<td>3 h</td>
<td>Luciferase/GFP labeling+bioluminescence/fluorescence microscopy</td>
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<td>Cardiac stem/progenitor cells</td>
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<td>Vrtovec et al; 2013</td>
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<tr>
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<td>Radiolabeling+single-photon emission CT</td>
<td>↑ LVEF</td>
<td>Vrtovec et al; 2013</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Patients with nonischemic</td>
<td>CD34+ stem cell</td>
<td>4.4±1.2</td>
<td>18 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>↑ LVEF</td>
<td>Vrtovec et al; 2013</td>
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<td>dilated cardiomyopathy</td>
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<tr>
<td></td>
<td>Patients with MI</td>
<td>Bone marrow mononuclear cells</td>
<td>17.3±6.2</td>
<td>4 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>↑ LVEF</td>
<td>Silva et al; 2009</td>
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<td>Patients with MI</td>
<td>Bone marrow mononuclear cells</td>
<td>10.6±6.1</td>
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<td>Radiolabeling+single-photon emission CT</td>
<td>↑ LVEF</td>
<td>Silva et al; 2009</td>
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<td>4.86 (1.7-7.6)</td>
<td>1 h</td>
<td>Radiolabeling+single-photon emission CT</td>
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<td>Musialek et al; 2011</td>
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<td>Systemic intravenous</td>
<td>Rat MI model</td>
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<td>1 d</td>
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<td>Wang et al; 2011</td>
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<td>Rabbit MI model</td>
<td>Bone marrow multilineage-</td>
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<td>3 d</td>
<td>GFP Labeling+fluorescence microscopy</td>
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<td>Yamada et al; 2018</td>
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<td></td>
<td>Pig I/R model</td>
<td>differentiating stress enduring cells</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Pig I/R model</td>
<td>Embryonic endothelial progenitor cells</td>
<td>0.5</td>
<td>1 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>Not significant</td>
<td>Kupatt et al; 2005</td>
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<td>Retrograde</td>
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<td>Mesenchymal stem cells</td>
<td>2.89</td>
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<td>Radiolabeling+nuclear imaging</td>
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<td>Gathier et al; 2019</td>
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<td>coronary venous</td>
<td>Pig MI model</td>
<td>Peripheral blood mononuclear cells</td>
<td>3.2±1</td>
<td>1 h</td>
<td>Radiolabeling+PET-CT</td>
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<td>Hou et al; 2005</td>
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<td>Embryonic endothelial progenitor cells</td>
<td>2.7</td>
<td>1 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>↓ Infarct size</td>
<td>Kupatt et al; 2005</td>
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<tr>
<td></td>
<td>Patients with MI</td>
<td>Bone marrow mononuclear cells</td>
<td>4.2±1.1</td>
<td>4 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>Not significant</td>
<td>Silva et al; 2009</td>
</tr>
<tr>
<td></td>
<td>Patients with MI</td>
<td>Bone marrow mononuclear cells</td>
<td>3.2±0.3</td>
<td>24 h</td>
<td>Radiolabeling+single-photon emission CT</td>
<td>Not significant</td>
<td>Silva et al; 2009</td>
</tr>
</tbody>
</table>
Table 1.2: Clinical and pre-clinical studies reported low retention of transplanted cells in the heart. Reproduced from Li et al.110
Generally, acute cell retention (1 hour after administration) in the heart is below 10%, regardless of cell type or administration route. 90% of the cells that are implanted die within the first week and <1% of administered cells are detectable after 4 weeks.

The number of viable cells transplanted to the heart sharply decreases after cell injection. To determine the mechanism by which the viability of transplanted cardiomyocytes acutely decreases after injectable administration, Dow et al. administered rat neonatal cardiomyocytes into the left ventricular wall of infarcted rats, at 15 min post-reperfusion or at 75 min post-permanent occlusion. Histological and PCR analysis determined that cardiomyocytes injected in the left ventricular wall of both treatment groups acutely washout from the infarct zone through the cardiac vasculature and into the lungs, liver, kidneys and spleen. Hence cell suspensions, cannot localize nor be retained to the target site of myocardial injury due to mechanical washout from the heart.

Given the low engraftment efficiency, cell therapy to date seems to have elicited its therapeutic effects through paracrine mechanisms, as discussed in section 1.2.4. Viable cells that are retained in the heart, can take residence, and exert long term therapeutic effects by the release of pro-survival paracrine factors. However, some of the discussed investigations in 1.2.5.2 have hinted to the production therapeutic effects via direct regeneration. Direct regeneration could be achieved through long term localized retention of mature CM cellular grafts via reduced graft death.

Hence, a major challenge in the clinical trials is delivery and retention of viable cells in the heart. If the cell therapy overcomes mechanical washout from the heart, it can engraft to the myocardium. After engraftment in the heart, the low retention of viable cells is likely to be associated with the harsh environment of the diseased heart tissue, as the cells delivered will be exposed to ischaemia and inflammation (Figure 1.12). Another reason for the lack of efficacy relates to the method used to process and deliver the cells to the patient, which disrupts pro-survival signals from the extra cellular matrix and triggers anoikis. Ischaemia, inflammation and anoikis account for the predominant mechanisms that contribute to engrafted cell death in the myocardium.
Figure 1.12: Potential mechanisms that might contribute to engrafted cell death in the ischaemic myocardium. Cytochrome C (C)(CytC). Created with BioRender.
1.3 Ischaemia

Injected cells will rely on perfusion from surrounding tissue to maintain their metabolic function until they engraft and can rely on delivery of oxygen and nutrients from the host vasculature. The physical limit of nutrient perfusion is ~150µm and this translates into limited graft diffusion\(^{119}\). Cellular grafts with cells deeper than 7-10 cell diameters, towards the middle of the graft, are at higher risk of becoming ischaemic\(^{119}\). The potential of ischaemic injury is exacerbated in myocardial injury, where the host tissue is ischaemic and the scarred tissue in the heart presents a hypoxic environment. The majority of injected cells implanted in the reduced-perfusion scarred tissue incur irreversible ischaemic injury within 24 hours of implantation, as shown by irregular mitochondrial matrix densities and severe cytoplasmic and mitochondrial swelling\(^{120}\). The ischaemic damage incurred by the cell graft follows a similar mechanism by which the host tissue was damaged by MI. Survival of transplanted neonatal CM has been shown to be directly proportional to tissue perfusion: the highest survival was seen in a healthy heart host, intermediate survival in a cardiac granulation tissue and the lowest survival in acutely necrotic cardiac tissue\(^{121}\). The damage is characterized by ATP depletion, initiation of anaerobic glycolysis with produced acidosis, dysregulation of ionic homeostasis, swelling attributed to osmotic load and ultimately membrane damage and cellular death\(^{122}\). Additionally, ischaemia leads to the formation of reactive oxygen species which increases mitochondrial permeability and subsequently exacerbates the risk of cellular damage\(^{123}\).

To overcome the hurdle of ischaemic death, numerous strategies have been explored in attempt to generate an enriched vascular supply and enhance graft survival. Pre-vascularization interventions have been utilized, followed by cell therapy. Pre-treatment of an infarcted region in rat hearts with the angiogenesis mediator, vascular endothelial growth factor (VEGF), followed by cell transplantation enhanced cellular cardiomyopathy. The efficacy of this treatment was attributed to the creation of a more favourable (vascularized) environment for transplanted cell survival\(^{124}\). Similarly, MSC were transfected to express VEGF and injected into an acute rat model of MI\(^{125}\). Over-expression of VEGF enhanced cell survival and improved cardiac function after 1 week of administration\(^{125}\). In a different approach, transplantation of adenoviral encoded hypoxia inducible factor 1alpha (HIF-1α)
skeletal myoblasts in a rat model of MI significantly increased angiogenesis, cell engraftment and cardiac function\textsuperscript{126}. HIF-1\(\alpha\) is a gene that controls the expression of numerous angiogenic factors which may have potentiated graft vascularization and/or pro-survival effects\textsuperscript{119,126}. Vascularisation has also been achieved by cell therapy. Pericyte transplantation has been shown to promote angiogenesis and reduce fibrosis in murine and swine models of MI\textsuperscript{21,72}.

Hence, ischaemia creates the hostile environment in the injured myocardium and characterizes the low-perfusion environment of the resultant infarct scar. Ischaemia is also one of the mechanisms which can hinder the success of cell therapy, but multiple approaches have been utilized to overcome this hurdle and to potentiate the functional benefits of cell transplantation.

1.4 Inflammation

Inflammation is an essential component to ischaemic heart injury and CHF, but also plays an essential role in the homeostatic functions of the heart. Immune cells infiltrate the myocardium before birth, where they reside and contribute to essential homeostatic function. A healthy adult mouse heart contains all of the major leukocyte classes: mononuclear phagocytes, neutrophils, B cells and T cells\textsuperscript{127}. A network of cardiac macrophages resides between cardiomyocytes, these macrophages are heterogeneous and ontogenically diverse. An inventory of cardiac macrophages was revealed using a murine model expressing green fluorescent protein controlled by Cxcr1 promoter\textsuperscript{128}. The dense macrophage network was profiled for surface markers, to reveal that there are at least 4 different subsets of cardiac macrophages. All the macrophages were CD45+F4/80+CD11b+CD64+MERTK+, but differed in expression of MHC Class II molecules, CC-chemokine receptor 2 (CCR2) and lymphocyte antigen 6C (Ly6C)\textsuperscript{128}. In addition to macrophages, small populations of other leukocytes reside in the heart. Tissue sections and single cell suspension of digested hearts indicate the presence of cardiac dendritic cells and mast cells\textsuperscript{129,130}.

The heart composition is not homogenous and the distribution of leukocytes is not uniform\textsuperscript{129,131,132}. The localization of immune cells in the heart suggests that the leukocytes have specific interactions with resident non-leukocytic cardiac cells\textsuperscript{133}. CM, fibroblasts, and endothelial cells produce cytokines, chemokines and growth factors which activate leukocyte response. Additionally, the cardiac cells express
receptors that can respond to products of activated leukocytes, including mast cell produced tumor necrosis factor which activates endothelial cells\textsuperscript{130} and T-cell produced IL17 which activates cardiac fibroblasts\textsuperscript{134}. Hence, the immune system participates in the stead-state physiology of the heart and the specificities of these contributions and interactions have been discussed in these detailed reviews\textsuperscript{133,135}.

After MI, in response to the ischemic injury, numerous innate and adaptive immune cells are recruited to the injured heart in order to remove necrotic tissue, scavenge pathogens and promote healing (Figure 1.13)\textsuperscript{133}.

![Figure 1.13 The progressive activation of immune cells at the MI zone, after ischaemic injury. Reproduced from Swirski & Nahrendorf\textsuperscript{133}. CC-chemokine receptor 2 (CCR2), vascular cell adhesion molecule 1 (VCAM), tumour necrosis factor (TNF), damage-associated molecular patterns (DAMPS), lymphocyte antigen 6C (Ly6C), lymphocyte antigen 6G (Ly6G), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), interleukin-10 (IL-10), macrophage-derived transforming growth factor-\(\beta\) (TGF-\(\beta\)). Shortly after ischaemic injury, resident mast cells release preformed granulocytes\textsuperscript{130}, resident macrophages and CM produce inflammatory cytokines and chemokines\textsuperscript{136,137}, fibroblasts release hematopoietic growth factors\textsuperscript{138} and endothelial cells activate to increase expression of adhesion molecules for further
leukocyte homing. These initial events induce the synthesis and recruitment of neutrophils and monocytes\textsuperscript{133}.

Recruited neutrophils and monocytes accumulate in the heart and participate in the inflammatory cascade. Initially, they scavenge necrotic material\textsuperscript{139}, but after 3 days neutrophils decrease in numbers and almost disappear after 7 days\textsuperscript{133,140}. In contrast, monocytes persistently accumulate at the ischemic region and differentiate to cardiac macrophages for numerous days\textsuperscript{141}.

Over the progression of several days, inflammation gives way to reparation. Neutrophils disappear driven by the decreased production of inflammatory cytokines, growth factors and chemokines\textsuperscript{142,143}. Reparative cardiac macrophages appear and initiate the production of tumor growth factor $\beta$ and VEGF which promote fibrosis and angiogenesis\textsuperscript{143}. The thinning and fibrosis of the myocardium leads to a decrease in ventricular contractility, which the heart compensates with hypertrophy, but the compensation mechanism eventually becomes pathological. Weeks after the ischemic injury, the coordinate inflammatory and reparative response of the numerous leukocytes subsides and the number of immune cells in the heart lowers back to homeostatic levels. The surviving myocardium remodels in an adverse manner which leads to varying degrees of CHF.

Studies have shown that the inflammatory and reparative response to MI must be precisely controlled to achieve the best resolution: the formation of a durable scar. An excessive accumulation of immune cells in the acute inflammatory phase, results in a thinning infarct that heals poorly\textsuperscript{144}. On the opposing end, a minimal acute inflammatory response results in the accumulation of excessive granulation tissue and the formation of an unstable scar\textsuperscript{145}.

The initial phase of inflammatory injury and repair has been well investigated, while the role of immune cells in the chronic phase of heart failure has been less explored. Analysis of human biopsies indicates that the number of blood monocytes and cardiac macrophages increases in HFpEF. Studies into mouse models of CHF, also indicate that the number of macrophages and leukocytes increase in the myocardium and have an altered phenotype. The inflammatory phenotypes of the immune cells in CHF alters their interaction with resident CM, endothelial cells and fibroblasts. In the ischemic myocardium, endothelial cells increase the expression of
adhesion molecules, such as E-selectin, for the recruitment of CD4 positive T cells which exacerbates fibrosis.

Lymphocyte-deficient mice\textsuperscript{146} and TCR\(\alpha\) knock out mice\textsuperscript{147} have a reduced fibrotic and hypertrophic response to aortic banding, which models increased cardiac afterload. Similarly, antibody-dependent T cell clearance reduced fibrosis and hypertrophy.

Mice with HFpEF induced by aldosterone treatment, nephrectomy and salt water diet increased the expression of pro-fibrosis IL-10 encoding gene in macrophages\textsuperscript{148}. The distribution of macrophage subset in the disease model was shifted towards the pro-fibrotic MHC class II\textsuperscript{hi} cells, which produce osteoponin and activate fibroblasts to enhance matrix production, while decreasing levels of metalloproteinases\textsuperscript{148}. Additionally, genetic deletion of \(IL10\) in macrophages, significantly reduced cardiac fibrosis and improve heart function\textsuperscript{148}.

The data discussed suggests that healing infarcts are highly inflammatory and chronic infarcts retain a pro-inflammatory immune cell phenotype. Leukocytes in the infarcted heart tissue release reactive oxygen species and inflammatory cytokines that contribute to the local harsh environment\textsuperscript{119}. The resultant inflammatory environment is likely to impede therapeutic cell engraftment and result in further loss of the transplanted cells. Acutely infarcted hearts are the most inflammatory, but this effect could also be considerable in the CHF setting, as macrophages acutely localise to grafted regions in healthy hearts\textsuperscript{119}. Injection of skeletal myoblasts with a free radical scavenger, superoxide dismutase, improved graft cell survival two-fold, 3 days post-administration\textsuperscript{149}. The effect was matched with an attenuated inflammatory response and subsequent reduced production of free radical species from pro-inflammatory leukocytes\textsuperscript{149}.

Aharinejad \textit{et al.} administered autologous skeletal myoblasts transfected with colony stimulating factor 1 (CSF-1) plasmid in the myocardium of a CHF rat model and showed improved cardiac function, superior to previous skeletal myoblasts cell therapies\textsuperscript{150}. CSF-1 is a cytokine expressed by multiple cells types and is a regulator of resident macrophage proliferation and survival\textsuperscript{151}. CSF-1 transfected skeletal myoblasts were administered via intramyocardial injection 3 weeks post MI induction, after the establishment of heart failure in the animals. Cardiac function was assessed
4 weeks and 9 weeks post MI, to establish that CSF-1 transfection enhanced myoblast proliferation and survival, which enabled improved cellular engraftment\textsuperscript{150}. Additionally, CSF-1 overexpression increased macrophage recruitment at the perimeter of the infarct area, which resulted in increased angiogenesis and matrix metalloproteinase 2 protein levels\textsuperscript{150}. These results translated into improved cardiac function and thicker infarcted wall thickness. Inhibition of CSF-1, has also been shown to attenuate resident cardiac macrophage proliferation 2 weeks after MI. The attenuation corresponded with an increased production of pro-inflammatory cytokines and poor cardiac function\textsuperscript{152}.

The data suggests that inflammation plays an essential role in the development and outcome of CHF. Leukocyte sub-set phenotype, in particular macrophages, are critical to the outcome of cell therapies and particularly to long-term graft survival.

1.5 Anoikis
Anoikis is one of the pathways that has been postulated to halt the efficacy of cell transplantation. Anoikis is the programmed cell death initiated in anchorage-dependent cells upon disruption of contact with the extra cellular matrix (ECM). The term was coined by Frisch in 1994, after identifying a form of apoptosis in epithelial tissue associated with detachment from the ECM.\textsuperscript{153} He noted that programmed cell death is crucial to maintaining regulated cell number, function and organization.\textsuperscript{153} However cellular sensitivity to anoikis could be regulated through genetic overexpression of bcl-2, viral manipulation by transformation with vHa-ras and v-scr and treatment with phorbol ester.\textsuperscript{153} The results suggested that alleviation of anoikis sensitivity results in the acquisition of anchorage independence and cellular metastasis.\textsuperscript{153}

To date, the importance of anoikis in maintaining homeostasis and regulating tumor metastasis is recognized and more recently the role of anoikis in the outcome of cellular transplantation has also been acknowledged.

Zvibel \textit{et al}. presented and popularized the importance of anoikis on the outcome of cell therapy, in particular in reference to liver transplant.\textsuperscript{154} Zvibel \textit{et al}. outlined the mechanisms underpinning the high rates of apoptosis during cell isolation, cryopreservation, administration and engraftment which hinder the therapeutic potential of cell therapy. Specifically, the importance of cellular pro-survival signaling
from β-integrin interaction with the ECM, the activation of caspase cascades upon disruption of ECM contact and the release of mitochondrial proteins to potentiate the actions of caspases. Ultimately, strategies to dampen anoikis and attain higher cell viability were proposed, to obtain higher cell engraftment efficiency and improve the outcome in liver cell transplantation.

To date the occurrence of anoikis in multiple cell types has been identified, in both primary and immortalized cell lines. To isolate single cell suspensions, calcium chelators and enzymes, such as collagenases, are utilized to detach the cells from the basement membranes. These isolation methods are likely to induce anoikis as soon as the cells detach from the matrix. A study found that isolating primary epithelial intestinal cells results in 90% apoptotic cell death if the culture was kept in suspension for 3 hours. Importantly, stem cells present in the crypt that express pro-survival bcl-2 were equally sensitive for anoikis. These findings suggest that anoikis presents a strong and rapid death signal, from which majority of cell types are susceptible, including the cell therapies utilised for heart regeneration.

Injectable cell therapies rely on the ideology that viable cells can take residence in the heart and exert long term therapeutic effects by the release of pro-survival paracrine factors. Cardiomyocytes and the other cell types used in the aforementioned clinical therapies, are adherent. In their natural state, the adherent cells are bound by integrins to the basal lamina. When cell-based therapies are processed for in vivo delivery, the cell component is typically detached from a substrate using enzymes and suspended in a buffer solution, often for many hours, prior to delivery to the patient via injection into the myocardium. Whilst in suspension, essential adhesion-dependent signals are lost until the cells are able to adhere to a substrate after implantation into the patient. Disruption of integrin ligands results in the cells losing adhesion-dependent pro-survival signals and anoikis. Interrupted integrin signalling activates mitochondrial cytochrome C release and leads to caspase-mediated cell death.

Animal and human clinical studies have demonstrated that low cell viability follows delivery of cell suspensions into the infarcted myocardium. In an infarcted porcine model, only 5% of injected mesenchymal stem cells survived for up to 14 days. Likewise < 0.5% of human mesenchymal stem cells were viable in mouse hearts at 4
days after transplantation\textsuperscript{161} and similar outcomes have been reported with transplantation of skeletal myoblasts, smooth muscle cells and unfractionated bone marrow cells\textsuperscript{149,162,163}. Human studies have also confirmed low cellular retention. Blocket \textit{et al.} reported the retention of 5.5\% peripheral-blood CD34+ cells, 1 hour after intracoronary administration in patients suffering from acute myocardial infarction\textsuperscript{164}. Hofmann \textit{et al.} reported 1.3-2.6\% retention of unselected bone marrow cells and 14-39\% retention of CD34-enriched cells post-administration\textsuperscript{165}. Generally, acute cell retention (1 hour after administration) in the heart is below 10\%, regardless of cell type or administration route\textsuperscript{110,113,114}. 90\% of the cells that are implanted die within the first week and <1\% of administered cells are detectable after 4 weeks\textsuperscript{110,115,116}.

1.5.1 Anoikis of CM

CM are also susceptible to anoikis and therefore to maximize the survival of transplanted CM in injectable therapies, approaches to circumvent anoikis need to be taken.

The survival of transplanted CM was first determined by Muller-Ehmsen \textit{et al.}\textsuperscript{116}. Murine male donor neonatal cardiomyocytes were administered into the left-ventricular wall of syngeneic female adult rats\textsuperscript{116}. Survival of the engrafted cardiomyocytes was quantified by TaqMan polymerase chain reaction and histology, to discern that 80\% of transplanted cells are lost within 24 hours of administration, independently of the number of cells administered\textsuperscript{116}. Caspase inhibitor AcYVADcmk did not improve cell survival at 24 hours, however, acutely, within 1 hour of transplantation, there was a positive relationship between the number of administered cells and cell survival\textsuperscript{116}. 15\% of transplanted cells survived up to 12 weeks and underwent maturation, with the development of mature sarcomeres\textsuperscript{116}. Grafted cells exhibited long-term survival up to 6 months, when administered in syngeneic female adult rats, 1 week after coronary artery ligation\textsuperscript{166}. Cardiac function was improved by thickening of the left ventricular wall, enhancing ejection fraction and reduction in dyskinesia\textsuperscript{166}.

Co-transplantation of mouse ESC derived-CM with mouse embryonic fibroblasts into the host myocardium synergistically improved cell engraftment\textsuperscript{167}. Histological analysis revealed that 24 hours after transplantation <1\% of cardiomyocytes and
50% of fibroblasts engrafted in the myocardium\textsuperscript{167,168}. The data suggests the importance of different cellular adhesive abilities and sensitivity to anoikis\textsuperscript{167,168}.

To improve the survival rate of administered cardiomyocytes, Laflamme \textit{et al.} injected differentiated cardiac-enriched hESC progeny into the left ventricular wall of athymic rats in conjunction with a pro-survival cocktail\textsuperscript{80}. Initial grafts were composed of predominantly non-cardiac cells which were lost overtime to obtain a predominant cardiomyocytes graft after 4 weeks\textsuperscript{80}. The administered graft increased in size threefold through heat-shock treatment, produced angiogenesis and enabled the proliferation of graft cardiomyocytes\textsuperscript{80}. In this strategy the presence of non-cardiac cell components may have acted as bridges to facilitate the engraftment of the cardiomyocytes to the host tissue and prevent the graft from washing out. Additionally, the pro-survival cocktail contained Matrigel (cell-permeable peptide from Bcl-XL which inhibits mitochondrial death signalling), cyclosporine A (dampens the activation of cyclophilin D-dependent mitochondrial signalling), pinacidil (model of ischaemic pre-conditioning) and IGF-1 (activates Akt and caspase inhibitor signalling)\textsuperscript{168}. The two component pro-survival strategy contributed to enhancing the engraftment and the viability of the administered cardiomyocytes.

Matrigel is a protein mixture derived from a murine sarcoma line and utilized as a commercially available extracellular matrix preparation. In pro-survival cocktails, the Matrigel can provide cells with mechanical attachment to mimic the ECM. Matrigel can synergistically work with the pro-survival chemicals to produce larger surviving grafts. However, the use of Matrigel in clinic is discouraged due to the presence of animal components and inconsistent batch variability. Alternatives to the use of Matrigel, for the provision of a pro-survival ECM to anchorage-dependent CM are under investigation. Ogasawara \textit{et al.} reproduced the study by Laflamme \textit{et al.}\textsuperscript{80}, but substituted Matrigel with a chemically defined hyularan based hydrogel, HyStem-C\textsuperscript{169}. The use of Matrigel and HyStem-C were specifically targeted to prevent anoikis. iPSC-CMs diluted in HyStem-C alone, a pro-survival cocktail with HyStem-C, or a pro-survival cocktail with Matrigel were delivered in a rat model of myocardial infarction\textsuperscript{169}. Histological analysis 4 weeks post-transplantations, revealed remuscularization of the infarct zone with iPSC-CM for all three groups\textsuperscript{169}. The group that received the Matrigel grafts, had the largest graft size and the grafted iPSC-CM had a more mature phenotype compared to the other treatments\textsuperscript{169}. Thus, hyularan-
based hydrogels do not provide a viable substitute for Matrigel, and further investigations are required to identify a clinically feasible pro-survival ECM substitute for CM transplantation.

The ECM is essential to cellular homeostasis and adhesion, in the heart it provides a scaffold for cellular anchorage, structure and supports contraction systems\textsuperscript{170}. The ECM is a complex micro-environment composed of proteins, glycoproteins, proteoglycans and proteins that contribute to the basement membrane\textsuperscript{170}. Given the importance of the ECM to the function of the heart and respective resident cells, it appears essential that CM for heart transplantation should be delivered on a pro-survival ECM to maximize graft survival and engraftment.

The importance and fragility of the ECM for cellular engraftment was demonstrated by McKown \textit{et al.}, showing that iPSC-CPCs showed a reduced ability to adhere to the ECM obtained from failing hearts \textit{in vivo} compared to the healthy control\textsuperscript{171}. The ECM of failing human hearts with dilated cardiomyopathy have an altered global protein content, which is associated with detrimental effects to the cells that it hosts and that can adhere to it\textsuperscript{172,173}. Despite the lack of adhesion, susceptibility to anoikis and blood clearance in ECM from failing hearts, McKown \textit{et al.} observed that the minority of cells which attached to the ECM did not show altered morphological development, beating and cardiac troponin T levels to the healthy control\textsuperscript{171}. Hence, despite the fragility and hostility of pathologically remodeled ECM, the study suggests that if we can promote adhesion of transplanted cells to the ECM of remodeled hearts, the graft has the potential to be therapeutic.

Overall, the research suggests that using approaches to circumvent anoikis can improve the survival and engraftment of transplanted CM. This is particularly important for improving the success of CM transplantation onto an unfavorable ECM in the diseased heart.

The survival of injected cells in pro-survival cocktails/viscous media\textsuperscript{80,169} has been superior to the survival of cells injected in liquid suspension\textsuperscript{116}. Despite, improved survival, the engraftment of these cells into the heart remains marginal\textsuperscript{163}. To improve attachment and engraftment of cell therapies, researchers are delivering cells via structured engineered constructs. However, application of structured engineered constructs requires open heart surgery and remains invasive\textsuperscript{174}. Hence,
of interest has been the use of biomaterials and tissue engineering approaches to provide a clinically feasible pro-survival ECM substitute for an injectable cell therapy.

1.6 Biomaterials and tissue engineering

As outlined above, the loss of cell viability associated with existing methods for delivering cells into myocardium is largely attributable to the loss of cell-substrate interactions, resulting in activation of apoptotic pathways. A simple way to address this problem would be to deliver the cells whilst anchored to a substrate. Biomaterials offer an ideal solution for achieving this. Biomaterials are designed to interact with a biological system for a therapeutic purpose - diagnosis, repair or replace. Biomaterials can be synthesized from natural (i.e., alginate and chitosan) or synthetic (i.e., polymers and metals) sources and be applied to a variety of different biological systems. Applications of biomaterials include polymeric drug delivery vehicles for cancer therapy\textsuperscript{175}, orthopaedic joint replacements\textsuperscript{176} and the development of contact lenses\textsuperscript{177}. Biomaterials must possess some critical properties independent of their application, including biocompatibility, lack of toxicity and mechanical properties. There are additional biomaterial properties that need to be considered dependent on the application of the biomaterial and these can be optimized by the selection of the composite material and the fabrication technique. These include reproducibility, size, porosity, surface morphology, mechanical strength, integration with the respective biological system and controlled degradability.

Biomaterials are an integral contributor to tissue engineering. Tissue engineering is a multidisciplinary field that applies engineering and life science approaches towards the development of novel therapeutics for the replacement, restoration, maintenance or improvement of a biological system\textsuperscript{178}. There are three components involved in tissue engineering (1) cells that can support the therapy through regeneration, remodelling and growth (2) a scaffold for transplantation and support (3) active molecules (i.e. cytokines and growth factors)\textsuperscript{179}. The three components of tissue engineering have been used individually or synergistically towards the development of novel therapeutics. Biomaterials provide a scaffold for cell adhesion and the creation of novel tissue. After implantation of the scaffolds \textit{in vivo}, cells can infiltrate the biomaterial and integrate with the recipient’s tissue. Implanted cells enhance integration of the recipients’ cells via cytokines and growth factor release.
engineering was initially used in 1970s for the generation of new cartilage using chondrocytes and eventual investigation into the development of tissue engineered skin substitutes by the 1980s\textsuperscript{180}. Vacanti and Langer paved the way to popularizing the subject in their Science Review in 1993\textsuperscript{181} and tested the feasibility of a biomaterial-based replacement of tracheal cartilage in 1994\textsuperscript{181}. To date, tissue engineering has been applied to numerous fields, including cardiovascular regeneration.

1.6.1 Cardiovascular applications

Biomaterials-based approaches for myocardial regeneration have utilized injectable hydrogels, preformed porous scaffolds, including cardiac patches made from electrospinning and 3D alginate scaffolds\textsuperscript{23,72,182–184}. Several different approaches have been investigated, as summarised in Table 1.3. More information on these studies can be found in these detailed reviews\textsuperscript{23,185}.

It is important to note that most of these biomaterials are intended as cell delivery vehicles and do not necessarily involve attachment of the cells to the material. Therefore, these approaches can be equivalent to delivering the cells in suspension. A novel and transformative approach would involve the use of an implantable substrate to which the cells are intended to adhere prior to delivery into the recipient tissue.
<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Formulation</th>
<th>Delivered cells</th>
<th>Proposed Mechanism</th>
<th>Application and Result</th>
<th>Advantages and Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGylated fibrinogen(^{186})</td>
<td>Hydrogel</td>
<td>Cardiomyocytes and Embryonic Stem Cells</td>
<td>Stabilization and strengthening of the scar, cellular integration to restore cardiac function</td>
<td>Improvement in fractional shortening</td>
<td>Biodegradable, increased cellular delivery via graft size, high porosity. Imunosuppression and requires optimization of material properties.</td>
</tr>
<tr>
<td>Chitosan(^{187})</td>
<td>Hydrogel</td>
<td>Brown adipose derived stem cells</td>
<td>Biomaterial increases collagen synthesis which enhances survival and differentiation of injected cells</td>
<td>Enhanced CM differentiation and increased angiogenesis</td>
<td>Integration of 25% seeded cells 4 weeks after implantation, therapeutic biomaterial. Limited source of brown adipose derived stem cells Chitosan obtained from animal source.</td>
</tr>
<tr>
<td>Chitosan(^{188})</td>
<td>Hydrogel</td>
<td>Adipose-derived mesenchymal stem cells</td>
<td>Reactive oxygen species scavenging, and recruitment of chemokines enhances cell engraftment and survival</td>
<td>Increased cell survival</td>
<td>Biomaterial itself was therapeutic and degraded after 4 weeks. Large number of engrafted cells died within the first week.</td>
</tr>
<tr>
<td>Fibrin glue(^{189})</td>
<td>Hydrogel</td>
<td>Adipose-derived stem cells</td>
<td>Paracrine mechanism, increase in vessel density, inhibition of apoptosis and/or enhanced myocardial elasticity</td>
<td>Improved heart function</td>
<td>Fibrin injections inhibited infarcted ventricle remodelling, big source of transplantable cells. Retention of cells was only slightly improved, and fibrin comes from animal origins.</td>
</tr>
<tr>
<td>Alginate-RGD(^{190})</td>
<td>Hydrogel</td>
<td>Mesenchymal stem cells</td>
<td>Induced angiogenesis arrested left-ventricular remodelling and further prevented wall thinning and chamber dilation</td>
<td>Reduced infarct size and enhanced cell survival</td>
<td>Combination therapy, RGD modification improved cell survival. Cells not detectable in situ after one day, microspheres not in uniform size.</td>
</tr>
<tr>
<td>PCL-Gelatine(^{174})</td>
<td>Electrospinning</td>
<td>Mesenchymal stem cells</td>
<td>Mechanical support and induction of angiogenesis.</td>
<td>Reduced scar size and microvessel formation</td>
<td>Biodegradable with suitable stiffness. Low cell engraftment.</td>
</tr>
<tr>
<td>PGS-Fibrinogen(^{191})</td>
<td>Electrospinning</td>
<td>Mesenchymal stem cells + VEGF</td>
<td>Biomaterial provides functional and structural support to the myocardium. Mesenchymal stem cells differentiated into cardiac cells for cardiac repair</td>
<td>Improved ejection fraction</td>
<td>Combination therapy. Differentiation into two types of cells, but not 100% efficient.</td>
</tr>
<tr>
<td>PLLA(^{192})</td>
<td>Knitting</td>
<td>Vascular smooth muscle cells</td>
<td>Improved elasticity of patches mediated beneficial effects</td>
<td>Improved left ventricular function</td>
<td>Biodegradable, highly porous material. Cells are easily obtainable and cultured. Cell seeding on the graft was not uniform, mechanical properties needed further testing and cell migration was not measured.</td>
</tr>
</tbody>
</table>

**Table 1.3: Overview of biomaterials used for cell therapy in cardiac regeneration.** Adapted from Hinderer *et al.*\(^{185}\) Polyethylene glycol (PEG), Arg-Gly-Asp peptide (RGD), polycaprolactone (PCL), poly(glycerol sebacate) (PGS), poly(l-lactic acid) (PLLA), cardiomyocytes (CM), vascular endothelial growth factor (VEGF).
Microcarriers are an efficient and effective technology for cellular applications. Microcarriers are microspheres of polymerized networks with a diameter of 100-400 µm, which have been optimized for cell attachment\textsuperscript{193}. Microcarriers have generally been developed for two cellular applications: (1) \textit{in vitro} cell expansion and biomolecule production (2) \textit{in vivo} cell delivery for regenerative medicine. Microcarriers can be easily manufactured with a high surface area to volume ratio. For \textit{in vivo} delivery, they can be administered via minimally invasive injections and localized to the target site\textsuperscript{194,195}. Microcarriers are designed and optimized to their application and the specifications of the anchored cell type\textsuperscript{194}. The specificity of the microcarrier, specifically the porosity, 3D microenvironment and degradability are customized by selection of the constituent polymer, crosslinking parameters, and post-synthetic modifications.

Natural and synthetic polymers have been utilised to produce cellularised microcarriers and each polymer has unique pros and cons. Natural polymers are typically low cost and biodegradable, like gelatin, however they suffer from batch specific variations and uncontrollable degradation\textsuperscript{193}. Synthetic polymers are biocompatible and easily tuneable, like polyethylene glycol (PEG), but are usually more expensive and require functionalization for cell adhesion\textsuperscript{196}.

The porosity of the microspheres determines the diffusion rate and the size of particulates going through the microcarriers\textsuperscript{197}. For immune protection, the pores of the microcarriers must prevent the infiltration of immune cells whilst maintaining metabolite diffusion\textsuperscript{198}. Microcarriers that require cellular infiltration, must be synthesized with bigger pores\textsuperscript{199,200}. These parameters can be attuned by coating mechanisms, polymer concentration and fabrication methodology.

The requirement for cell-adhesive functional groups on the microcarriers is dependent on the cell type of interest. Anchorage dependent cells require adhesion to prevent anoikis. Synthetic polymers do not have cell-adhesive functional groups and therefore require functionalization by blending, coating, or attaching cell-adhesive moieties. These moieties can be whole or truncated ECM proteins, adhesion polymers or synthetic protein fragments, that are recognised by integrins on the cell surface\textsuperscript{194,201,202}. 
Some polymers are degradable *in vivo* through hydrolysis or weakening of the cross-linked networks\(^{193,203}\). In contrast, covalently cross-linked polymers do no weaken and are not degradable\(^{193}\). Ideally, for tissue regeneration the materials should be biodegradable, with the degradation products naturally metabolised by the host and negating the need to remove the material post-administration.

The microcarrier fabrication technique is determined by the desired microcarrier size, size, scalability, and ease of the process\(^{204}\). There are a variety of techniques available, with respective advantages and disadvantages. Common microcarrier fabrication techniques are illustrated in Figure 1.14, including emulsion formation, extrusion formation, atomization, and microfluidics.

**Figure 1.14: Schematics of commonly used microcarrier fabrication techniques:** (A) Emulsion formation, (B) extrusion formation; atomization and setting of solvents by (C) air drying solvent extraction or (D) polymerization in a collection bath and (E) microfluidics on a T junction plate. Created with BioRender.
Microcarriers are produced by emulsion formation (Figure 1.14 A) by addition of a polymer solution to a stirred un-mixable continuous phase and polymerization occurring in the stirred container\textsuperscript{194,204,205}. The mechanical stirring breaks the polymer solution into droplets. Extrusion of a polymer solution (Figure 1.14 B) via a nozzle is collected into a continuous phase container where polymerization occurs\textsuperscript{206}. Atomization of polymer drops can occur by setting via solvent extraction (Figure 1.14 C)\textsuperscript{207} or setting of chemically-responsive polymers (i.e. temperature, ion or photo-dependent cross-linking) (Figure 1.14 D) in a collection bath\textsuperscript{208}. Microfluidics on a T-junction (Figure 1.14 E) contain the two immiscible polymer and continuous phases, flowing at the same rate, producing continuous pinching off droplets from the dispersed polymer solution\textsuperscript{200}. More information on these techniques, their advantages and disadvantages can be found in this detailed review\textsuperscript{193}.

Cellularised microcarriers have been utilized for cardiovascular applications, as summarized in Table 1.4. There is a limited number of studies on the application of cellularised microcarrier for myocardial regeneration. Majority of the published studies target cardiovascular function thorough pro-angiogenic paracrine factors released from cellularised grafts, rather than cell delivery and implantation. These were further limited by immunogenicity, xenogeneic graft components and marginal cell engraftment. Hence, there is a clear need to investigate an appropriate cellularised microcarrier that will enhance cellular engraftment to potentiate myocardial regeneration therapy.
<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Formulation</th>
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<th>Proposed Mechanism</th>
<th>Application and Result</th>
<th>Advantages and Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA205</td>
<td>Microcarriers coated in laminin</td>
<td>Stromal vascular fraction cells</td>
<td>Tissue repair factors secreted from the cells have pro-angiogenic effects and decrease necrosis.</td>
<td>Streptozotocin-induced diabetic mice underwent hind limb ischaemia. Improved blood flow and reduced necrosis.</td>
<td>Biodegradable, sterilised through gamma irradiation, high porous microenvironment and can encapsulate growth factors. Immunosuppression, microcarriers need size exclusion, graft size and survival were not quantified.</td>
</tr>
<tr>
<td>Alginate198</td>
<td>Microcapsules containing BaSO4</td>
<td>Mesenchymal stem cells</td>
<td>Arteriogenesis and angiogenesis through paracrine mechanisms or ischaemic preconditioning mechanisms.</td>
<td>Improve hind limb perfusion in a rabbit model of peripheral arterial disease.</td>
<td>Semipermeable membrane protects against immunorejection but permits diffusion. Can be imaged for biocompatibility and efficacy with CT. Some inflammatory infiltrate and no MSC survival. Contrast agent obscures the presence of neo-vessels.</td>
</tr>
<tr>
<td>PLGA200</td>
<td>Microfluidic device fabricated spherical porous beads</td>
<td>Human amniotic fluid stem cells</td>
<td>Engrafted cells produced neovascularization and myocardial regeneration through differentiation into endothelial and smooth muscle cells. Differentiation and secretome secretion improved cardiac and contractile function.</td>
<td>Induction of cells into angiogenic and cardiomyogenic lineages, engraftment of cells and beads into a rat model of myocardial infarction and improving cardiac function.</td>
<td>Uniform physical size of biodegradable beads with large pores. Cells retains pluripotent beads and release endogenous ECM. Use of animal components for cell culture, requirement for immunosuppression.</td>
</tr>
<tr>
<td>Gelatine209</td>
<td>Beads</td>
<td>Human umbilical vein endothelial cells</td>
<td>Production of paracrine factors dependent on growth characteristics and shear stress.</td>
<td>In vitro: production of large-scale culture of functionally active endothelial cells for various vascular applications. Can provide model into shear street.</td>
<td>Biodegradable microcarriers that can support large scale culture of endothelial cells. Can provide controlled secretome release according to shear stress. Cells proliferate on carrier Animal components and some chromosomal aberration.</td>
</tr>
</tbody>
</table>

**Table 1.4: Overview of microcarriers used for cardiovascular applications.** Poly(lactic-co-glycolic acid) (PLGA), barium sulphate (BaSO₄), extracellular matrix (ECM), computed tomography (CT), matrix metallopeptidase 2 (MMP2), mesenchymal stem cells (MSC).
1.6.2 Key attributes of an ideal implantable cellularised substrate

Based on the cell-based therapies and biomaterial investigations conducted to date for treatment of cardiovascular disease it is possible to identify several key attributes required by the implantable substrate for delivering cells in an anchored state. These include:

- Biocompatibility with the cells
- Retention of pluripotency for stem cells and their differentiation into target cells
- Compatible with minimally invasive delivery
- Achieve cell retention at the target site
- Evidence of longer-term viability and engraftment with host tissue
- Biodegradation into non-toxic products

Of particular interest in this project, was the utilization and optimization of poly(lactic-co-glycolic acid) (PLGA) Thermally Induced Phase Separation (TIPS) microspheres as an implantable substrate for regenerative cell delivery to the failing heart.

1.6.3 PLGA TIPS microspheres

Polymers are chosen to create biomaterials that interact with cells, because they are biocompatible and easily customized for the intended application. This thesis focused on the use of PLGA for the fabrication of microcarriers. PLGA are a family of medically approved biodegradable polymers that have strong physical bonds and are highly biocompatible. PLGA (Figure 1.15 A) is a co-polymer of poly-lactic acid (PLA) (Figure 1.15 B) and poly-glycolic acid (PGA) (Figure 1.15 C). PLGA is highly tuneable, it is possible to tune the physical properties of the polymer matrix by controlling the polymer’s molecular weight and ratio of PLA and PGA. Tuning these parameters can optimize the polymer’s degradation rate, mechanical strength, and porous morphology, for the intended application.
Figure 1.15: Chemical structures of (A) poly(lactic-co-glycolic acid) [PLGA], (B) monomer of poly-lactic acid [PLA] and (C) monomer of poly-glycolic acid [PGA]. x and y refer to the relative amount of PLA and PGA units. Carbon (C), oxygen (O), hydrogen (H). Reproduced from Sequeira et al. 211

PLGA degrades when exposed to moisture or aqueous environments (Figure 1.16). PLGA is biologically hydrolysed into lactic and glycolic acid212. These hydrolysis products are endogenously metabolised by the body via the Krebs cycle and ultimately cleared from the body as carbon dioxide and water212. The degradation rate of PLGA can be controlled by adjustment of the monomer ratios within the polymer213. PLA is composed of more methyl groups and this renders it more hydrophobic than PGA213. The presence of more methyl groups results in a lower water absorption in the polymer and therefore slower degradation of the ester bonds213. Hence, polymers with a higher PLA to PGA ratio, such PLGA 85:15, will degrade slower than PLGA 10:90.

Clearing of PLGA has minimal systemic toxicity as the hydrolysis products are endogenously removed, leaving no trace at the end, but only increased local acidity due to the degradation214. This may produce irritation at the site of polymer implantation214.
Figure 1.16: Hydrolysis of PLGA into lactic and glycolic acid, which are endogenously metabolised by the Krebs cycle. Poly(lactic-co-glycolic acid) (PLGA), carbon (C), oxygen (O), hydrogen (H), repeats of lactic acid co-polymer unit (x), repeats of glycolic acid co-polymer unit (y), number of PLGA co-polymers (n), carbon dioxide (CO₂) and water (H₂O). Reproduced from Sequeira et al.²¹¹

The mechanical strength of PLGA is affected by molecular weight, polydispersity index and degree of crystallinity of PLGA²¹³. This is further dependent on the type and molar ratio of the monomer components in the copolymer chains. PLA can be made in a highly crystalline form (poly L-lactic acid) or in a completely amorphous form (poly D-lactic acid) with disordered polymer chains²¹⁵. PGA has a highly crystalline form due to the absence of methyl groups²¹³. A higher degree of crystallinity results in more rigid monomer chain structures which produce a co-polymer with stronger mechanical strength²¹³,²¹⁵.

PLGA has shown great potential as a biomaterial, where it has shown numerous biological applications, including as a drug delivery vehicle²¹⁶. PLGA microcapsules
have been formulated to deliver particulates, antibodies\textsuperscript{217} and active molecules\textsuperscript{218,219}. PLGA has also shown potential for tissue engineering applications. Bible \textit{et al.} delivered neural stem cells on PLGA microparticles for neuro-integration, \textit{de novo} tissue formation and vascularisation in the lesion cavity of a murine stroke mode\textsuperscript{195,218}. Rocha \textit{et al.} delivered VEGF containing PLGA microcapsules into an engineered intestine construct which enhanced epithelisation via increased angiogenesis and sustained VEGF release\textsuperscript{220}. Ahmadi \textit{et al.} utilised PLGA microcarriers for delivery of smooth muscle cells for treatment of faecal incontinence\textsuperscript{221}. The utilization of PLGA microcarriers also displayed superior cell attachment and delivery in comparison to gelatine microcarriers\textsuperscript{221}.

Biodegradable PLGA microspheres have been produced through a variety of methods which present their unique advantages and disadvantages. Water-oil-water emulsion methods have been used for the encapsulation of hydrophilic proteins and drugs. However, this method generally poorly controls microsphere size and size distribution, with typical mean diameter deviations of 25-50\% off the target size\textsuperscript{222}. The spray drying technique enables the encapsulation of a wide range of particulates in a reproducible manner, however the technique is low yielding and poorly controls size distribution\textsuperscript{213,223}. Microfluidics has been utilized to provide a superior control over particle production, including shape and size, as well as providing a more integrated system, utilizing a single step emulsification for the multiple components\textsuperscript{224}. On the other hand, the technique is time consuming due to the production of a single drop per time and therefore is not suitable for scaling up\textsuperscript{213}. Ultimately, each fabrication technique, along with its unique advantages and disadvantages will be chosen depending on the desired characteristics of the microsphere and its target application.

Cellularised microcarriers for tissue engineering should be porous, with a characterized pore size and an interconnected framework to enable vascularization. The TIPS technique can formulate microcarriers with these desired properties. The structure and the porosity of the microcarriers can be determined through the adjustment of the fabrication parameters. These parameters include, choice of polymer, polymer concentration, solvent to non-solvent ratio, quenching rate, and cooling rate. As a result, adjustment of the fabrication parameters can produce a
wide range of porous structures with specific morphologies tailored to their application\textsuperscript{203,225}.

The TIPS process involves the preparation of a mixed and homogenous polymer and organic solvent solution. The polymer solution is mixed either exposed to an immiscible solution or cooled below its solubility temperature. The process utilizes thermal energy to induce phase separation, which separates the solution into a polymer-rich and solvent-rich phase\textsuperscript{203,226}. The solution gets cooled to the target quenching temperature utilising a ramp temperature rate\textsuperscript{203}. The typical temperature-concentration phase diagram of a polymer solution is composed of two curves: a bimodal and spinodal curve (Figure 1.17)\textsuperscript{203}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{The temperature concentration phase diagram of polymer solutions during TIPS formulation. Reproduced from Martínez-Pérez et al.\textsuperscript{203}. Temperature (T), concentration (Φ), region (R).}
\end{figure}

The spinodal curve represents spinodal decomposition, the development of two co-existing microphase separated material from one thermodynamic phase. The spinodal curve divides the process into two regions, the stable and unstable phases. The binodal curve defines the limit of solvent solubility. When the temperature of a solution is above the binodal curve, there is a homogenous polymer solution. The merged peaks of the binodal and spinodal curves represent the critical point of the solution\textsuperscript{203}. During TIPS, the polymer solution passes from the homogenous single
(stable) phase region into the spinodal (unstable) region of the binary phase diagram through a rapid change of solution temperature, this fast transition is called a quench\textsuperscript{227}. The quench enables phase transition to move from nucleation and growth into spinodal decomposition (Figure 1.18)\textsuperscript{203,227}. As the polymer enters into the spinodal region, it becomes unstable and undergoes spontaneous separation into polymer-rich and solvent-rich regions\textsuperscript{203,226,228}. Removal of the solvent-rich regions via freeze-drying or freeze-extraction produces the final porous structure\textsuperscript{225}.

![Figure 1.18: The biphasic elements of nucleation and spinodal decomposition. Adapted from Ashlesha & Kalonia\textsuperscript{229} and Nam & Park\textsuperscript{228}.](image)

TIPS fabrication enables the production of porous and interconnected scaffolds through manipulation of TIPS parameters:

1. Polymer type

The choice of polymer for a given application is based on numerous criteria, including material chemistry, molecular weight, solubility, hydrophilicity/hydrophobicity and degradation profile\textsuperscript{225}. As previously discussed, polymers can be synthetic or natural, each type with its unique advantages and disadvantages.

Table 1.5 compares the properties of different synthetic and natural polymer scaffolds produced with the TIPS techniques.
Table 1.5: The properties of different polymeric scaffolds fabricated with by TIPS.
Adapted from Akbarzadeh & Yousefi. Poly(l-lactic acid) (PLLA), poly (lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), polyurethaneurea (PU).

Interestingly, PLLA and PLGA are the most used polymers for TIPS fabrication. Zhang et al. reported that scaffolds made from PLLA and hydroxyapatite (HA) (Figure 1.19A) had different pore morphologies compared to PLGA/HA scaffolds (Figure 1.19B) fabricated with the same TIPS parameters. PLGA/HA scaffolds had a higher density and smaller pores due to increased shrinkage of PLGA/HA scaffolds during freeze drying. Molecular re-arrangement of the polymer scaffolds during shrinking was higher in PLGA due to the amorphous nature of the polymer which is less stable than crystalline PLLA. Hence, different polymers can produce different morphologies under the same experimental conditions.
Figure 1.19: SEM micrographs of PLLA/HA scaffold (50/50) at (Ai) x100 and (Aii) x500 magnification, and PLGA/HA scaffold (50/50) at (Bi) x100 and (Bii) x500 magnification fabricated with the same TIPS parameters. Reproduced from Zhang et al. 230.

(2) Polymer concentration

Polymer concentration effects the porous morphology of the material produced via TIPS. The polymer concentration can affect the liquid phase separation to produce bundles of channels or ladder-like structures due to the preferential orientation created by the crystallization of the solvent. 236

Polymer concentration can also impact the dominance of polymer-rich or solvent-rich phase. 237 Reducing polymer concentration increases porosity and reduces the material density (Figure 1.20) 230. However, when polymer concentration falls below a critical level, the solvent-rich phase becomes dominant to the polymer-rich phase. 237 Upon removal of the solvent, the dispersion of polymer-rich droplets through the solvent-rich matrix causes the structure to collapse. 237
Figure 1.20: SEM micrographs of PLLA/HA scaffolds (50/50) at (i) x100 and (ii) x500 magnification, prepared from different polymer concentrations: (A) 1% PLLA/dioxane (w/v), (B) 5% PLLA/dioxane (w/v), (C) 7.5% PLLA/dioxane (w/v). Reproduced from Zhang et al.230

(3) Solvent/non-solvent ratio

The solvent system can impact reaction efficiency, porous morphology, and mechanical properties of the porous material. A mixture of solvent/non solvent system is more efficient than a mono solvent system238. Wei and Ma showed that by using a dioxane/water mixture to prepare PLLA/HA solutions, liquid-liquid phase separation was more efficiently induced238. The proportion of water in the solvent system greatly reduced pore size, increased pore size distribution, altered pore
morphology from ladder-like to random and affected the connection of the porous network (Figure 1.21)\textsuperscript{238}. Materials fabricated with a mixed solvent system also displayed lower mechanical strength, measured by compressive modulus\textsuperscript{238}. In mono-solvent systems, the materials produced have regular and orientated pore structures, but with mixed solvent systems the pore structure is random\textsuperscript{238}. This reduces the compressive modulus of the materials\textsuperscript{225,238}.

Figure 1.21: SEM micrographs of PLLA/HA scaffolds (70/30) prepared from different solvent systems: (A) dioxane/water 95/5 \( \times \)500 magnification, dioxane/water 90/10 (Bi) \( \times \)500 (Bii) \( \times \)8000 magnification., dioxane/water 87/13 (Ci) \( \times \)45 (Cii) \( \times \)500 (Ciii) \( \times \)10000 magnification. Reproduced from Wei & Ma\textsuperscript{238}. 

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(4) Quenching temperature and time

Quenching route is another TIPS parameter that controls the morphology of TIPS products. As previously mentioned, quenching enables the transition from nucleation and growth, into spinodal decomposition. Because tissue engineering favours materials with open porous networks and inter-connection pores, it is preferred to quench under the spinodal curve, where liquid-liquid separation takes place by spinodal decomposition\(^{203}\). On the other hand if quenching in the metastable region, liquid-liquid phase separation occurs in the metastable region via nucleation and growth mechanisms and the materials produced will have a poorly connected and beady morphology\(^{228}\). Lower quenching temperatures lead to faster cooling rate and a shorter time for solvent nucleation, solvent crystallization and phase separation (Figure 1.22B)\(^{237}\). Therefore, the TIPS products will have smaller pores. Higher quenching temperatures favours larger solvent crystals which produce TIPS products with larger pores (Figure 1.22A)\(^{239}\).

![SEM micrographs](image_url)

Figure 1.22: SEM micrographs of PLLA/HA scaffolds (50/50), 2.5% (w/v) PLLA/HA/dioxane, at (i) x100 and (ii) x500 magnification, prepared from different quenching temperatures: (A) 8°C and (B) liquid nitrogen. Reproduced from Zhang et al\(^{230}\).
(5) Coarsening

The parameters of polymeric molecular weight, concentration, quenching route, type of solvent and additives greatly influence the TIPS products at the early stage of phase separation\(^{203}\). At the later stage of phase separation, phase separated droplets continue to coalesce in order to minimize the interfacial free energy that is associated with the interfacial area\(^{203}\). This process is called coarsening. Coarsening is induced by the differential interfacial tensions created by the two phase separated regions\(^{203}\). It has been demonstrated that coarsening results in TIPS products with larger pore sizes and thus this process should be considered to control the product morphology (Figure 1.23)\(^{228}\). However, the coarsening process tends to produce more closed pores (Figure 1.23C) and therefore it is important optimize the other TIPS parameters in order to fabricate TIPS products with an interconnected and open pore structure\(^{203}\).

Figure 1.23: SEM micrographs of PLGA scaffolds, 9% (w/v) polymer solutions, coarsening over time at 4°C: (A) 0 minutes, (B) 2 minutes and (C) 10 minutes, followed by quenching in liquid nitrogen. Reproduced from Nam & Park\(^ {228}\).

Adjusting the TIPS parameters has enabled the creation of porous constructs with distinctive morphologies tailored for numerous applications. The TIPS process can fabricate nano-, micro- or macro-particles, fibres, polymer films and polymer coatings\(^ {240}\). TIPS processed polymers have been used in a range of tissue engineering applications, including scaffolds for bone-tissue engineering\(^ {230,231}\), cellular scaffolds for soft-tissue regeneration\(^ {233,234}\) and stem cell differentiation\(^ {241}\). Zare-Mehrajdi et al. successfully differentiated stem cells into neural cells, on PLLA TIPS microparticles\(^ {241}\). By comparing stem cell survival and differentiation on TIPS microparticles to 2D cell culture, the group found that stem cells successfully infiltrated the TIPS microparticles expressed increased neural differentiation markers and favourable neurite outgrowth\(^ {241}\).
TIPS fabrication has also been applied to the formulation of PLGA TIPS microspheres with specified and monodispersed size distributions\textsuperscript{217}. A PLGA / dimethyl carbonate (DMC) polymer solution was sprayed through a nozzle with acoustic vibrations to produce controlled droplets\textsuperscript{217}. The droplets were collected in a liquid nitrogen (quencher) container for liquid-liquid phase separation of the PLGA polymer and DMC solvent\textsuperscript{217}. The solvent was consequently removed through freeze drying\textsuperscript{217}. The formulation of microspheres through this technique enables precise control of the porosity and size distribution of the product, which have been problematic with emulsion fabrication\textsuperscript{217,222}. The technique also overcomes complications over repeatable particle production and diminishes exposure to the aqueous continuous phase, which were associated with other microsphere fabrication techniques, such as rapid solvent removal via temperature gradient\textsuperscript{242}, gas foaming\textsuperscript{243}, double emulsification\textsuperscript{244} and solution-induced phase separation\textsuperscript{245}.

The PLGA TIPS microspheres have previously been shown to be an excellent substrate for the cellular attachment of skeletal myoblasts, mesenchymal stem cells and numerous other cell types\textsuperscript{246}. Additionally, the microspheres have been shown to integrate and retain \textit{in vivo} after implantation. The fabrication process for TIPS microspheres is flexible and it can be scaled-up for clinical use. However, to date it is not known whether these materials are suitable for delivery to cardiac tissues, what impact they have on the behaviour of iPSC and differentiation into cardiomyocytes. Or whether delivery of cells attached to TIPS microspheres provides a novel type of cell microcarrier that improves the potency of proposed cell-based therapies.
1.7 Hypothesis and aims

The hypothesis of this thesis is as follows: ‘TIPS microspheres provide a suitable substrate for attachment, growth and \textit{in vivo} delivery of iPSC-CM that will facilitate myocardial tissue regeneration by the enhanced engraftment of viable iPSC-CM’.

To address this hypothesis, the aims of the project were:

1. Determine a suitable TIPS microsphere formulation for iPSC attachment.
2. Optimise the TIPS microsphere formulation to support iPSC culture and phenotype.
3. Determine a suitable delivery formulation for the injection of TIPS microspheres.
4. Demonstrate the safety of delivering TIPS microspheres \textit{in vivo}.
5. Formulate a composition of TIPS microspheres that can be tracked \textit{in vivo} without causing unwanted side-effects for cell delivery using the microcarriers.
6. Demonstrate that iPSC attached to TIPS microspheres retain their required biological behaviour.
7. Demonstrate that iPSC attachment to the TIPS microspheres protects from anoikis.
8. Justify the selection of iPSC-CM cardiac differentiation protocol and parameters.
9. Demonstrate that iPSC-CM attached to TIPS microspheres retain the target phenotype.
10. Finalise the delivery formulation for the injection of cellularised TIPS microspheres.
11. Demonstrate that TIPS microspheres improve the engraftment and viability of iPSC-CM in a healthy preclinical rodent model.

Aim specific objectives are detailed in the relevant chapters.
Chapter 2: Materials and methods

2.1 Fabrication of TIPS microspheres

2.1.1 Polymer solutions

To prepare the polymer solution of 2% (w/v) PLGA, 0.8 g PURASORB PDLG 7507, 75:25 DL-lactide/glycolide copolymer (Corbion Biomaterials) was dissolved in 40 ml dimethyl carbonate (DMC) (anhydrous >99%, D152927, Sigma Aldrich) in 50 ml Falcon tubes (352070, BD Biosciences). 2.0g and 4.0 of PURASORB PDLG 7507 were dissolved in 40 ml DMC in 50 ml Falcon tubes, to prepare 5% and 10% (w/v) solutions respectively. Samples were mixed using magnetic stirring overnight at room temperature (20 °C).

For preparation of TIPS microspheres loaded with barium sulphate (BaSO\textsubscript{4}) (10442031, Fisher Scientific), 0.4, 1.2 and 8 g BaSO\textsubscript{4} was added to 40 ml of the 2% polymer solution and magnetically stirred for an additional 1 hour at room temperature (20 °C) to obtain 2%, 6% and 20% BaSO\textsubscript{4} (w/v) loaded polymer solution.

For preparation of TIPS microspheres loaded with indocyanine green (ICG) (A10472, Adoq Bioscience) a 1 mg/ml solution was prepared by dissolving 10.8 mg of ICG in 10.8 ml of absolute ethanol (32221-2.5L-M, Sigma Aldrich). 1 ml of ICG solution was added to 19 ml of the 2% polymer solution and stirred for an additional 1 hour at at room temperature (20 °C) to obtain 10% ICG (v/v) loaded polymer solution.
2.1.2 TIPS microsphere processing

![Diagram of the TIPS microsphere fabrication equipment. Created with BioRender.]

The polymer solutions were passed into Var D Nisco encapsulation unit (Nisco Engineering) and ejected through a sapphire tipped nozzle with a 150 µm opening using a syringe pump (Harvard apparatus) at a flow rate of 2.2 ml/min. The nozzle vibrated at a frequency of 1.80 kHz with 70% frequency amplitude. Resulting polymer droplets were collected into a polyethylene beaker (10349324, Fisher Scientific) filled with 500 ml of liquid nitrogen to induce phase separation. To ensure that the residual DMC did not dissolve the polymer coating by reaching melting temperature (2-4 °C) the samples were transferred to a -80 °C freezer prior to transfer to a freeze-drier (Edwards Freeze-dryer EF03, Edwards). Once transferred to a freeze drier the samples underwent lyophilisation for 18 hours, enabling the sublimation of any residual DMC solvent. Batches of the lyophilised TIPS microspheres were sieved to size ranges of <250 µm, 250-425 µm, 250-355 µm, 355-425 µm and 425-500 µm using stainless steel sieves ((ndecottsTM stainless steel test sieve, Fisher Scientific).
2.1.3 Hydrophilisation of the microspheres

500 ml Ham's F-10 nutrient mix (ThermoFisher 11550043) was supplemented with 20% fetal bovine serum (FBS) (10500064, Life Technologies), 1% L-glutamine, 1% antibiotics/antimycotics (A5955, Thermo Fisher), 48 µl dexamethasone (D4902, Sigma-Aldrich) and 120 µl of 50 µg/ml stock human FGF-basic (154 a.a.)(100-18B-100UG, PeproTech).

20 mg of TIPS microspheres were weighed in a 7 ml polystyrene container.

A wetting solution for the hydrophilisation of TIPS microspheres was prepared, composed of 5 ml complete Ham's F10 and 1 ml 70% ethanol (E10600/05, Fisher Scientific).

For ICG coated TIPS microspheres, an ICG in dH2O solution was made by dissolving 10 mg of ICG in 10 ml of dH2O. An ICG in 70% ethanol solution was made by dissolving 10 mg of ICG in 10 ml of 70% ethanol. The wetting solutions were prepared, composed of:

1. 5 ml of ICG in dH2O solution and 1 ml 70% ethanol
2. 5 ml dH2O and 1 ml ICG in 70% ethanol solution
3. 5 ml complete Ham's F10 and 1 ml ICG in 70% ethanol solution

The wetting solution was transferred to the 7 ml polystyrene container containing 20 mg of TIPS microspheres, the vial was closed with the screw cap and wrapped in parafilm (BRND701611, VWR). The vial was vortexed (Vortex Genie 2) for 10-15 seconds at 1000 rounds per minute (RPM) and the samples were incubated in a hybridization oven for 72 hours at 37 °C under constant rotation at 30 RPM.

2.1.4 Pre-conditioning TIPS microspheres to maximise iPSC attachment

2.1.4.1 Selection of the polymer composition for the TIPS microspheres

0.1cm^3 of 2%, 5% and 10% 7507 TIPS microspheres sized 250-425 µm were measured in a 7 ml polystyrene container and 4 containers per polymer composition were prepared. The microspheres were hydrophilised as described in section 2.1.3.

2.1.4.2 Selection of a wetting solution to render the TIPS microspheres hydrophilic

To select the optimal wetting solution that would enable maximum iPSC attachment to the TIPS microspheres, a screen of 6 solutions containing different cell media and protein controls, was investigated. 20 mg of TIPS microspheres were weighed in a 7
ml polystyrene container and 24 containers were prepared. The following wetting solutions were prepared:

1. 20 ml Essential 8 media (A1517001, Thermo Fisher) and 4 ml 70% ethanol
2. 20 ml complete Essential 8 media and 4 ml 70% ethanol
3. 20 ml complete Ham’s F10 media and 4 ml 70% ethanol
4. 20 ml complete Ham’s F10 media 4 ml 70% ethanol and 5 µg/ml VTN-N (A14700, Thermo Fisher)
5. 20 ml HBSS, 4 ml 70% ethanol and 5 µg/ml VTN-N
6. 20 ml HBSS, 4 ml 70% ethanol and 5 µg/ml albumin bovine serum (BSA) (A7030-100G, Sigma-Aldrich)

To investigate the effect of FBS concentration on the sinking speed of the microspheres, solutions containing 20 ml HBSS, 4 ml 70% ethanol and 5-40% FBS were prepared.

6 ml of wetting solution was transferred to the 7 ml polystyrene container containing the TIPS microspheres, the container was closed and wrapped in parafilm. The container was vortexed at 1000 RPM for 10-15 seconds and the samples were incubated in a hybridization oven for 3-14 days at 37 °C under constant rotation at 30 RPM.

2.1.4.3 Selection of a protein coating composition for the TIPS microspheres

VTN-N was selected to coat the surface of the TIPS microspheres, however the optimum coating concentration needed to be established. The following solutions were made:

1. 10 ml HBSS, 2 ml 70% ethanol (0 µg/ml VTN-N)
2. 10 ml HBSS, 2 ml 70% ethanol and 5 µg/ml VTN-N
3. 10 ml HBSS, 2 ml 70% ethanol and 10 µg/ml VTN-N
4. 10 ml HBSS, 2 ml 70% ethanol and 15 µg/ml VTN-N
5. 10 ml HBSS, 2 ml 70% ethanol and 20 µg/ml VTN-N

20 mg of TIPS microspheres were weighed in a 7 ml polystyrene container and 8 containers were prepared. 6 ml of wetting solution was added to each container and the solutions were investigated in technical duplicates. The vials was closed and wrapped in parafilm. The vial was vortexed at 1000 RPM for 10-15 seconds and the samples were incubated in a hybridization oven for 14 days at 37 °C under constant rotation at 30 RPM.
2.1.4.4 Selection of a protein coating method for the TIPS microspheres

In addition to selecting a protein coating composition, the method of coating the TIPS microspheres was evaluated. This was used determine which method would result in the highest iPSC attachment.

The direct coating method provides protein coating at the same time that the microspheres undergo hydrophilisation. For this method, 20 ml complete Ham’s F10 media, 4 ml 70% ethanol and 5 µg/ml VTN-N wetting solution was prepared.

The post-wetting coating method, enables the TIPS microspheres to hydrophilize first, followed by coating for 1 hour in a concentrated protein solution. For this method 20 ml complete Ham’s F10 media and 4 ml 70% ethanol wetting solution was prepared.

20 mg of TIPS microspheres were weighed in a 7 ml polystyrene container and 8 containers were prepared. 6 ml of wetting solution was added to each container, the container was closed and wrapped in parafilm. The containers was vortexed at 1000 RPM for 10-15 seconds and the samples were incubated in a hybridization oven for 24 hours at 37 °C under constant rotation at 30 RPM.

After 24 hours, the containers containing the post-wetting coating solution were rinsed three times with 2 ml 1X Ca$^{2+}$/Mg$^{2+}$ free DPBS (D8357, Sigma).1 ml of 5 µg/ml VTN-N coating solution was added to each vial for 1 hour.

2.1.5 Delivery formulation of TIPS microspheres for in vivo delivery

TIPS microsphere were suspended into gel formulation in order to produce a homogenous microsphere suspension for in vivo delivery. To produce this suspension, hydrophilised microspheres were washed three times with 1 ml of Essential 8 Media and re-suspended in 500 µl - 1 ml solution of 20-60% (v/v) GRANUGEL® (Convatec) in dH$_2$O. 200 µl of 60% GRANUGEL® and microspheres suspension were loaded in a 250 µl Hamilton® syringe (20703, Sigma-Aldrich) or 1 ml B Braun™ Injekt™-F fine dosage syringe (10303002, Fisher Scientific), and capped with a 23 G/20 mm/pt style 4 needle (RE55167, Thames Restek).
2.2 Physical characterization of the microspheres

2.2.1 Characterisation of the morphology of TIPS microspheres during processing

10 mg of 2% 7507 TIPS microspheres of size range <250 μm, 250-355 μm, 355-425 μm and 425-500 μm were weighed in separate 7 ml polystyrene containers. A wetting solution composed of 40 ml Hank’s balanced salt solution (HBSS) (14060040, Thermo Fisher), 8 ml 70% eEthanol and 5 µg/ml VTN-N was prepared. 6 ml of wetting solution was added to each container, the container was closed and wrapped in parafilm. The containers were vortexed at 1000 RPM for 10-15 seconds and the samples were incubated in a hybridization oven for 14 days at 37 °C under constant rotation at 30 RPM.

On day 1, 3, 5, 7 and 14 of wetting, 200 µl of wetting solution containing the microspheres was removed. 100 µl of wetting solution transferred to a 12 well plate, the media was removed using a pipette tip firmly pressed to the bottom of the plate, and the microspheres were imaged using a Zeiss Primovert light microscope running Zen Blue Software. The remaining 100 µl of wetting solution transferred to a 48 well plate, the media was removed, the microspheres were washed with 300 µl of distilled water and air dried for 7 days for SEM analysis.

After 14 days of wetting, the wetting solution was removed and exchanged for 6 ml of HBSS to simulate the effect of degradation on the TIPS microspheres. The 21-day time point was selected to reflect the length of the cardiomyocyte differentiation protocol. The containers were closed, wrapped in parafilm and incubated in a hybridization oven for 21 days at 37 °C under constant rotation. On day 7, 14 and 21 of degradation, 200 µl of wetting solution containing the microspheres was removed. 100 µl was transferred to a 12 well plate for imaging and the remaining 100 µl was transferred to a 48 well plate for SEM analysis.

The microsphere diameter and circularity were characterised using ImageJ.

2.2.2 Microsphere sizing

The number of microspheres loaded in the Hamilton® (20703, Sigma-Aldrich) for in vivo delivery was quantified with an automated static image analysis system (Morphologie G3 microscope; Malvern). 20 mg of wetted 2% 7507 TIPS microspheres were resuspended in 1 ml solution of 60% GRANUGEL®. The microsphere formulation was loaded in a 1 ml syringe capped with a 23 G/20 mm/pt
style 4 needle. 3 boluses of 50 µl per sample were injected on a glass slide (631-1551, VWR) and covered with a glass cover slip (AGL463450, Agar Scientific). The slides were placed on a 4-slide holder chamber and analysed using a Standard Operating Procedure to automatically count the microspheres in suspension. Post-measurement, the count was manually validated. 4 independent biological samples, composed of 12 bolus injections, were analysed.

2.2.3 Ultrastructural imaging using scanning electron microscopy (SEM)
TIPS microspheres were mounted on aluminium discs using adhesive carbon tabs, coated with 1-2 nm of gold/palladium compound for 3 minutes in the Argon chamber of a high resolution ion beam coater (Gatan model 681). The TIPS microspheres were then analysed with Jeol 7401 high resolution field emission scanning electron microscope (SEM) at a range of magnifications (x400 - x5000).

EDS elemental mapping was performed on the same microscope.

2.2.4 Protein attachment to the hydrophilized microspheres
Protein adsorption to the surface of the microspheres was measured using a MicroBCA assay (23235, Thermo Scientific). 20 mg of 2% 7507 TIPS microspheres of size range <250 µm, were weighed in separate 7 ml polystyrene containers. Wetting solutions composed of phenol free HBSS (14175095, Thermo Fisher) were prepared:

(1) 15 ml HBSS, 3 ml 70% Ethanol (0 µg/ml VTN-N)
(2) 30 ml HBSS, 6 ml 70% Ethanol and 5 µg/ml VTN-N
(3) 15 ml HBSS, 3 ml 70% Ethanol and 5% FBS
(4) 15 ml HBSS, 3 ml 70% Ethanol and 10% FBS
(5) 15 ml HBSS, 3 ml 70% Ethanol and 20% FBS

6 ml of wetting solution was added to each container, the container was closed and wrapped in parafilm. The containers was vortexed at 1000 RPM for 10-15 seconds and the samples were incubated in a hybridization oven for 14 days at 37 °C under constant rotation at 30 RPM.

On day 0, 1, 2, 4, 6, 11, 12, 13 and 14 of wetting, 200 µl of wetting solution containing was removed and collected in low protein binding microtubes (72.706.700, Starstedt), sealed and stored at 4 °C. After 14 days of wetting, the
wetting solution was removed and the microspheres were washed with 1 ml of phenol free HBSS, for a total of three times. After washing, the microspheres were transferred to 1.5 ml low protein binding microfuge tube and resuspended in 150 µl of phenol free HBSS.

150 µl of previously collected media was transferred to a 96 well plate and the MicroBCA assay was run using the microplate method. BSA standards were prepared according to the manufacturer’s instructions. 150 µl of each standard solution was added to the 96 well plate. MicroBCA working reagent was prepared by mixing 5.5 ml reagent A, 5.28 ml reagent B and 220 µl reagent C together. 150 µl of working reagent was added to each well and microfuge tube prepared. The solutions were mixed thoroughly for 30 seconds on a plate shaker. The plate was covered using sealing tape, the microfuge tubes were capped and incubated at 37°C for 2 hours. After the 2 hours, the samples were cooled at room temperature (20°C). 300 µl of the solution in the microfuge tube was transferred to the 96 well plate. The absorbance of all samples was measured at 562 nm on a Versamax plate reader (210988, Biomax). A standard curve was prepared by plotting the average blank corrected 562nm reading of each protein standard against its concentration (Figure 2.2). The standard curve was used to determine the protein concentration of each test sample.

![Standard curve](image)

**Figure 2.2:** Standard curve of blank-corrected 562 nm readings of BSA standards.
2.3 Determination of TIPS microsphere biocompatibility in vitro

2.3.1 L929 cell culture

Cell culture was performed in class II laminar flow hoods using aseptic technique. For all cytotoxicity experiments, L929 immortalised mouse fibroblast at passage 10 were used. Cells were maintained DMEM (D5796, Sigma), 10% standard FBS, 1:100 diluted non-essential amino acids (100X stock), 1:100 diluted L-glutamine (100X stock), 1:100 diluted Penicillin/Streptomycin (100X stock). The frozen vial of L929 was thawed in a 37 °C water bath, transferred to a 15 ml conical tube and 10 ml of complete DMEM media was slowly added drop-wise to the cells. The cell suspension was centrifuged at 200 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 10 ml of pre-warmed DMEM media, transferred to a T75 flask, swirled to disperse the cells and placed into a 37 °C 5% CO₂ incubator, and incubated overnight. The next day, the media was replaced with 10 ml fresh supplemented DMEM, which was subsequently replaced every two days.

Cell growth was monitored using light microscopy and passaged once cells reached 70-80% colony confluency. The culture medium was removed using a 10 ml sterile serological pipette (CC114-4101, Appleton Woods) without disturbing the monolayer. 5 ml of pre-warmed PBS (P5493-1L, Sigma) was added to the flask using a 5 ml sterile serological pipette (CC109, Corning) to wash the cells and removed. 5 ml Trypsin-EDTA (T3924, Sigma,) was warmed to room temperature (20°C) and added to the flask. The flask was closed and placed back into the incubator. After 5 minutes, the flask was removed from the incubator and cellular detachment was monitored using a light microscope. The flask was transferred to the laminar flow hood and 5 ml of complete DMEM media was added to neutralise the Trypsin. The solution was transferred into a sterile 30 ml centrifuge tube (60.9922.212, Sarstedt) and centrifuged at 200 x g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of complete DMEM media. To determine cell count and viability a NucleoCounter NC-200 automated cell counter (ChemoMetec) was utilised. 200 μl of cell suspension was loaded into ChemoMete Via1-Cassette™ (941-0002, ChemoMetec) containing acrinide orange and DAPI. The cassette was loaded into the NucleoCounter and cell count and viability were determined to establish seeding densities. 5x10^3 cells/ 200 μl were seeded in a 96-well plate and expanded overnight for the LDH assay.
2.3.1.1 LDH assay
1 ml of complete DMEM was added to 20 mg of wetted 2% 7507 TIPS microspheres (negative control), 46.87 mg of 2% 7507 20% BaSO4 TIPS and conditioned overnight under dynamic conditions, under constant rotation at 30 RPM. 100 µl of conditioned media or complete media (vehicle control) was added to 1×10^5 L929 cells and toxicity was assessed 1, 2, 3 and 7 days after exposure using CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780, Promega). Media was replaced every two days. No cell control, vehicle control and maximum LDH release control were also included. For the LDH release control, 10 µl of 10X Lysis Solution (per 100µl original volume) was added to the positive control wells 45 minutes before the addition of CytoTox 96® Reagent. After 45 minutes, 50 µl aliquots from all test and control wells were transferred to a fresh 96-well plate. 50 µl of the CytoTox 96® Reagent was added to each sample and incubated at room temperature (20°C) in the dark for 30 minutes. 50 µl of stop solution was added to each well, bubbles were popped using a needle and the absorbance was measured at 490 nm within 10 minutes of adding the stop solution. LDH release was corrected to percent cytotoxicity using the following formula:

\[
\text{Percent cytotoxicity} = 100 \times \frac{\text{Experimental LDH Release (OD}_{490})}{\text{Maximum LDH Release (OD}_{490})}
\]

2.3.2 Induced-pluripotent stem cell culture
Human Episomal iPSC line was purchased from Thermo Fisher (A18945, Thermo Fisher). iPSC culture was performed in class II laminar flow hoods using aseptic technique. iPSC were maintained in in Essential 8 medium and for all experiments iPSC between passages 4-10 were used. A T75 flask was coated with VTN-N at a concentration of 0.5 µg/cm^2 for 1 hour in the laminar flow hood. The vitronectin solution was removed from the flask. Essential 8 Media and the Essential 8 Media supplement were pre-warmed at room temperature (20°C). 10 ml of supplement was added to 500 ml of media. The frozen vial of iPSC was thawed in a 37 °C water bath, transferred to a 15 ml conical tube and 10 ml of complete Essential 8 media was slowly added drop-wise to the cells. The cell suspension was centrifuged at 200 x g for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 8 ml of pre-warmed Essential 8 media and transferred to the VTN-N coated flask. 80 µl of ROCK inhibitor RevitaCell™ Supplement (A26445, Thermo Fisher) at 1X final
concentration was added to the flask, swirled to disperse the cells and placed into a 37 °C 5% CO₂ incubator, and incubated overnight. The next day, the media was replaced with 10 ml fresh complete Essential 8, which was subsequently replaced daily.

Cell growth was monitored using light microscopy and passaged once they reached 70-85% colony confluency. The culture medium was removed using a 10 ml sterile serological pipette without disturbing the monolayer. 5 ml of DPBS was added to the flask using a 5 ml sterile serological pipette to wash the cells and removed. 5 ml of gentle dissociating enzyme Tryple (12605010, Thermo Fisher) was warmed to room temperature (20°C) and added to the flask. The flask was closed and placed back into the incubator. After 5 minutes, the flask was removed from the incubator and cellular detachment was monitored using a light a microscope. The flask was transferred to the laminar flow hood and 5 ml of complete Essential 8 media was added to neutralise the Tryple. The solution was transferred into a sterile 30 ml centrifuge tube and centrifuged at 200 x g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of complete E8 media. Cell count and viability was determined using the NucleoCounter NC-200 as described in section 2.3.1. A split ratio of 1:3 -1:5 was used dependent on colony appearance and growth rate. After expansion, iPSC were cultured in 6 well tissue culture plates (140675, Thermo Fisher).

2.3.2.1 iPSC transduction with eGFP and firefly luciferase

iPSCs were transduced with lentivirus to express luciferase for BLI imaging, with the help of Dr. Stephen Patrick, CABI, UCL. Lentivirus encoding firefly luciferase and eGFP was produced in HEK 293 T cells using calcium phosphate precipitation protocol adapted from Tiscornia et al., using the transfer plasmid pSEW-Flagx3-FLuc-2A-GFP, together with packaging plasmids, Gag-pol (pCMV-R8.74; Addgene Plasmid #22036) and VSV-G (pMD2.G; Addgene Plasmid #12259). To improve viral titres, sodium butyrate (1mM) was added to the media, 24 hours prior to lentiviral harvest. Lentivirus was harvested into iPSC culture medium (Complete E8 media), passed through a 20 µm syringe filter, and added directly to P5 iPSCs for transduction. The remaining virus was transferred to 7 ml bijou tubes and stored at -20°C. Successful transduction was confirmed by eGFP expression using fluorescence microscopy (EVOS FL Auto cell imaging system, ThermoFisher
Scientific). After 24 hours iPSC were fed with fresh media and cultured for 4 days. After expansion, eGFP positive cells were selected using fluorescence activated cell sorting as described in section 2.3.6.3.2.

2.3.3 Cardiomyocyte culture

2.3.3.1 Differentiation of induced-pluripotent stem cells into cardiomyocytes

iPSC-CM were obtained by differentiation of iPSC at 30% colony confluency, using PSC Cardiomyocyte Differentiation Kit (A29212-01, Thermo Fisher). On day 0, a 6 well plate was coated with 0.5 µg/cm² VTN-N for 1 hour at room temperature (20°C) in the laminar flow hood. Confluent iPSC were split and cell numbers were quantified. Excess VTN-N coating solution in the 6 well plate was discarded and 2 ml of Essential 8 media supplemented with 20 µl of Revitacell was added to each well. 4x10⁴ cells were plated in each well and the plate was transferred into a 37 °C 5% CO₂ incubator. 24 hours after iPSC seeding, the spent media was discarded and the cells were fed with 2 ml of fresh E8 media and returned to the incubator. Cell growth was monitored daily, and the cells were fed until the colony confluence reached 30%, around day 4 of culture. Once iPSC exhibited the desired confluence, differentiation day 1 commenced. The spent media was discarded and replaced with 2 ml of pre-warmed cardiomyocyte differentiation medium A (A229209-01, Thermo Fisher) into each well. The plate was returned to the incubator for 48 hours. On differentiation day 3, the spent media was discarded, and 2 ml of pre-warmed cardiomyocyte differentiation medium B (A229210-01, Thermo Fisher) was added to each well. The plate was returned to the incubator for 48 hours. On differentiation day 5, the spent media was discarded, and 2 ml of pre-warmed cardiomyocyte maintenance medium (A229208-01, Thermo Fisher) was added to each well. The plate was returned to the incubator and fed every 48 hours with fresh cardiomyocyte maintenance media. Contracting cardiomyocytes were visible from day 6-8 of differentiation and maintained in culture using cardiomyocyte maintenance medium up to 40 days.

iPSC-CM detachment was performed using the Tryple method described in section 2.3.2. For cells later than 15 days, 0.5 U/ml Liberase TH (05401151001, Roche) and 50 U/ml DNase I (18047-019, Life Technologies) were added to the TrypLE dissociation solution to break down deposited collagen and facilitate detachment.
2.3.3.2 Differentiation of iPSC-CM on TIPS Microspheres

iPSC (day 0 of differentiation), partially differentiated iPSC-CM (day 2 or 4 of differentiation), early iPSC-CM (day 6 differentiation) and late iPSC-CM (day 18 of differentiation) were attached to the microspheres as described in section 2.3.3.1. Cells were attached and incubated in E8 for iPSC, Media A for day 2 iPSC-CM, Media B for day 4 iPSC-CM and maintenance media for day 6 and 18 iPSC-CM, for 2 hours post-seeding. After cell attachment, the differentiation process was continued, by change of media, up to day 20 as described in section 2.3.3.1.

2.3.3.3 Cardiomyocyte Purification

2.3.3.3.1 Metabolic selection

Metabolic selection and cardiomyocyte purification was based on the method by Burridge et al.94 iPSC-CM were metabolically selected from day 10 to day 16 of cardiomyocyte differentiation. CDM3 supplement was prepared by adding 0.4224 g of L-ascorbic acid 2-phosphate (49752-10G, Merck) to 20 ml water for injection (LZBE17-724F, Scientific Laboratory Supplies), inverting intermittently. The solution was mixed until clear. 1 g of recombinant human albumin (SC-OsrHSA-10, Caltag) was added, thoroughly mixed and filter sterilized. The supplement was divided into 500 µl aliquots in 1 ml microtubes and store at −20 °C. Lactate enriched CDM3 (CDM3-L) was prepared by supplementing 50 ml RPMI 1640 no glucose (11879020, Thermo Scientific), with 500 µl of CDM3 aliquot and 4mM L-lactic acid (0215393110, MP Biomedicals). On day 10, the spent cardiomyocyte maintenance media was discarded and replaced with 2 ml of pre-warmed CDM3-L. CDM3-L was replaced every 2 days. On day 16, the media was replaced with cardiomyocyte maintenance media and replaced every 2-4 days.

2.3.3.3.2 Magnetic selection

After cardiac differentiation, iPSC-CM were isolated using the PSC-derived CM Isolation Kit (130-110-188, Miltenyi Biotec) and LS separation columns (130-042-401, Miltenyi Biotec).
Figure 2.3: Two step magnetic separation strategy for the isolation of iPSC-CM. Step 1 depleted non-CM from the mixed iPSC-CM population, (A) non-CM in the mixed cell population were labelled with magnetic beads, (B) were passed through a magnetic separator to remove the labelled cells, (C) leaving the enriched iPSC-CM fraction. Step 2 enriched the iPSC-CM population, (D) CM in the enriched iPSC-CM fraction were labelled with magnetic beads and (E) passed through a magnetic separator. (F) Magnetically isolated cells were flushed out from the column to collect the purified iPSC-CM. Created with BioRender.

The following buffer and media were prepared ahead of the separation:

Isolation Buffer: Ca\textsuperscript{2+}/Mg\textsuperscript{2+} free DPBS, 0.5% BSA, 2 mM EDTA (15575-020, ThermoFisher). Filter-sterilised using a 0.2 μm filter.

EB 20% Media: Knockout DMEM (10829018, ThermoFisher), 20% HyClone-defined FBS (10703464, ThermoFisher), 1:100 diluted stock non-essential amino acids (11140035, ThermoFisher), 1:100 diluted stock L-glutamine, 45.4 μM β-Mercaptoethanol (31350-010, ThermoFisher), 1:100 diluted stock Penicillin/Streptomycin.
**EB 2% Media**: DMEM (D5796, Sigma), 2% standard FBS, 1:100 diluted stock non-essential amino acids, 1:100 diluted stock L-glutamine, 45.4 μM β-mercaptoethanol, 1:100 diluted stock Penicillin/Streptomycin.

Following the preparation of the solutions, iPSC-CMs were washed twice with DPBS and dissociated using the Multi Tissue Dissociation Kit 3 (130-110-204, Miltenyi Biotec) as per the manufacturer’s instructions. Briefly, enzyme T was diluted 1:10 with buffer X, and 600 μL of the enzyme mix was added to each well and incubated for 10 minutes at 37°C, 5% CO₂. Following incubation, 1.2 ml of EB 20% media was added to each well, and cells were detached by pipetting up and down. Cell suspensions were filtered using a 70 μm cell strainer (CLS431751, Corning) into a 50 ml tube. Each well was washed once with EB 20% media, which was then filtered and added to the final cell suspension. Cell counting was performed as described previously in section 2.3.1 to determine isolation cocktail volumes. The cell suspension was centrifuged at 200 g for 5 minutes, and cells were resuspended in 80 μl isolation buffer per 7×10⁶ cells. For each 7×10⁶ cells, 20 μl of the non-CM depletion cocktail was added, mixed, and then incubated for 5 minutes at 2-8°C. Cells were then washed by adding 1 ml isolation buffer per 7×10⁶ cells and centrifuged at 200g for 5 minutes. Supernatant was aspirated, and cells resuspended in 80 μl isolation buffer per 7×10⁶ cells. 20 μl of anti-biotin microbeads were added per 7×10⁶ cells, before mixing and incubation for 10 minutes at 2-8°C. During this incubation, LS separation columns were placed in the magnetic field of a QuadroMACS separator (130-091-051, Miltenyi Biotec) and prepared by washing with 3 ml isolation buffer. Following incubation with the anti-biotin microbeads, 400 μl of isolation buffer was added per 7×10⁶ cells, and 500 μl of cell suspension was added to each LS separation column, with flow-through collected in a 15 ml tube. LS separation columns were then washed 3 times with 3 ml of isolation buffer. Unlabelled cells were collected in a 15 ml tube, which represents the enriched iPSC-CM fraction.

The enriched iPSC-CM fraction was centrifuged at 200g for 5 minutes, and the cell pellet resuspended in 80 μl isolation buffer per 7×10⁶ cells. 20 μl of CM enrichment cocktail was added per 7×10⁶ cells, mixed, and incubated for 10 minutes at 2-8°C. During this incubation, new LS separation columns were placed in the magnetic field.
of a QuadroMACS separator and prepared by washing with 3 ml isolation buffer. Following incubation with the enrichment cocktail, 400 µl isolation buffer was added per 7×10⁶ cells, and 500 µL of cell suspension was added to each LS separation column. LS separation columns were then washed 3 times with 3 ml of isolation buffer, and the flow through collected in a 15 ml tube. During this step the iPSC-CMs were positively selected, retained in the magnetic column and were not collected in the flow-through. Therefore, the LS column was removed from the magnetic field, 5 ml of isolation buffer was added to the LS separation column, and the magnetically labelled cells were flushed in to a new 15 ml tube using the plunger provided.

Following isolation, iPSC-CMs were centrifuged at 200g for 5 minutes and resuspended in 1 ml pre-warmed (37°C) EB 2% media supplemented with 1x RevitaCell. Cell counts were performed, and the suspension was diluted to a concentration of 100,000 cells/200 µl for monolayer attachment or 600,000 cells/500 µl for microsphere attachment as described in section 2.3.4.2.
2.3.4 Cell attachment to TIPS microspheres and detachment

2.3.4.1 Attachment of iPSC

20 mg of hydrophilised and coated TIPS microspheres were washed with 2 ml of Essential 8 media, 3 times. The microspheres were resuspended in 500 µl of Essential 8 and transferred to a 24 well low bind cell culture plate (CLS3527, Sigma). $5 \times 10^5$ iPSC were suspended in 500 µl of Essential 8 and added to each well containing the microspheres, to obtain a total volume of 1 ml. The plate was incubated at 37 °C 5% CO$_2$ on a plate shaker (SciQuip Microplate mixer), shaking intermittently for 10 seconds every hour at low speed (~100 rpm), for 24 hours to maximise cell interaction with the microspheres and attachment.

2.3.4.2 Attachment of iPSC-CM

20 mg of hydrophilised and VTN-N coated TIPS microspheres were washed with 2 ml of CM maintenance media, 3 times. The microspheres were resuspended in 500 µl of CM maintenance media and transferred to a 24 well low bind cell culture plate. 0.6-1×10$^6$ iPSC-CM were suspended in 500 µl of CM maintenance media and added to each well containing the microspheres, to obtain a total volume of 1 ml. The plate was incubated at 37 °C 5% CO$_2$ on a plate shaker, shaking intermittently for 10 seconds every hour at low speed (~100 rpm), for 24 hours to maximise cell interaction with the microspheres and attachment.

2.3.4.3 Cell detachment from TIPS microspheres

A dissociation solution was prepared consisting of Tryple, 0.5 U/ml Liberase TH (05401151001, Roche) and 50 U/ml DNase I (18047-019, Life Technologies). Spent media from the cellularised microsphere samples was discarded and the samples were washed 3 times with 1 ml DPBS. 1 ml of dissociation solution was added to each sample and incubated at 37 °C 5% CO$_2$ for 5 minutes. After the first incubation, the sample and larger aggregates were disrupted by pipetting up and down 10 times using a P1000. The sample was returned to the incubator for another 5 minutes. After the final incubation, the sample was pipetted up and down a further 10 times to dislodge the cells from the microspheres. Using a Pasteur pipette, the sample was filtered through a 40 µm cell strainer (352340, Scientific Laboratory Supplies) to separate the cells from the microspheres. The cell flow through was collected in 15 ml conical tube and 1 ml of E8 or CM maintenance media was added to the sample.
The sample was centrifuged at 200g for 5 minutes. Supernatant was aspirated, and cells resuspended in media or processed for further experiments.

2.3.5 Quantification of cell attachment to TIPS microspheres
To determine the number of cells attached to the TIPS microspheres and their viability, the NC-200 automated cell counter was utilised. The microspheres were rinsed in the 24 well plate with 500 µl pre-warmed fresh DPBS, three times, to remove non-attached cells. Using a 1 ml Pasteur pipette, the contents of each well were transferred into a 1.5 ml microfuge tube (E1415-1500, StarLab). The microspheres were allowed to settle for 1 minute. The supernatant was removed using a 1 ml micropipette, ensuring that the microcarriers were not removed. The tip of the pipette was firmly pressed into the bottom of the microfuge tube to draw up the supernatant, leaving the microspheres. 300 µl of fresh, Essential 8 media or CM maintenance media, was added to the 1.5 ml microfuge tube containing the microcarriers. 300 µl of lysis buffer (Reagent A) (910-0003, ChemoMetec) were added to the sample and vortexed at 1000 RPM for 5 seconds to mix. The solution was left to incubate for 1 min at room temperature (20°C). 300 µl of stabilization buffer (Reagent B) (910-0002, ChemoMetec) were added to the sample and vortexed at 1000 RPM for 5 seconds to mix. 200 µl of cell suspension was loaded into ChemoMetec Via1-Cassette™. The cassette was loaded into the NucleoCounter and cell count and viability were determined to measure the TIPS microspheres biocompatibility with iPSC and iPSC-CM.

2.3.6 Evaluation of TIPS microsphere attachment on cellular phenotype
To establish whether attachment of iPSC and iPSC-CM to the TIPS microspheres retained their expected phenotypes, immunocytochemistry analysis of pluripotent and cardiac markers, and the differentiation studies were performed. 20 mg of 2% 7505 TIPS microspheres were weighed into a 7 ml polystyrene container and 4 containers were weighed out. The TIPS microspheres were wetted according to the method described in section 2.2.4 using solution (2). iPSC and iPSC-CM were attached to the TIPS microspheres as described in section 2.3.4.
2.3.6.1 Microscopical evaluation

2.3.6.1.1 Alkaline phosphatase assay

Pluripotency of the iPSC was evaluated using the StemAb Alkaline Phosphatase Staining Kit II (00-0055, ReproCell), a routinely used assay as high levels of alkaline phosphatase are a marker of pluripotency. A wash solution of 0.05% Tween 20 was prepared, transferring 10 ml of PBS (P5493-1L, Sigma Aldrich) in a 15 ml conical tube and adding 5 µl of Tween 20 (P1379, Sigma). AP substrate solution was prepared by mixing 1 ml of solution A with 1 ml of solution B in a 7 ml polystyrene container. The solution was left to incubate at room temperature (20°C) for 2 minutes. 1 ml of solution C was added to the complete AP substrate solution. The 24 well plate containing cellularized microspheres from section 2.3.6 was transferred to the laminar flow hood. The spent media was discarded, and each well was washed with 500 µl of wash solution. 500 µl of 4% in PBS (4% PFA) (12777847, Fisher Scientific) solution was added to each well and the cells were fixed for 5 minutes. The PFA solution was removed, discarded and the wells were washed with 500 µl of wash solution. 600 µl of AP substrate solution was added to each well. The plate was sealed in parafilm and stored in the dark at room temperature (20°C) for 10 minutes. After the incubation, the substrate solution was removed, and each well was washed twice with 500 µl of PBS. The wells were filled with 1 ml of PBS and alkaline phosphatase expression was immediately analysed using Zeiss Primovert Light Microscope running Zen Blue Software.

2.3.6.1.2 Confocal microscopy

Confocal microscopy was performed using an Olympus TIFR inverted microscope equipped with Fluoview software, or Zeiss LSM 980 confocal with Airyscan 2 equipped with Zen Blue software. Images were processed using Fiji software.

2.3.6.1.2.1 Sample preparation

200 µl of cellularised TIPS microsphere suspension prepared in 2.3.4.1 and 2.3.4.2 were transferred into separate wells of an 8 well chambered coverglass (155409PK, Thermo Fisher).

Monolayer samples were grown directly on the 8 well chambered coverglass. The Coverglass was coated with 5 µg/ml VTN for 1 hour at room temperature (20°C) and the cells were cultured as described in section 2.3.2 and 2.3.3.
2.3.6.1.2.2 General cellular markers
The distribution of cell attachment on the TIPS microspheres was evaluated using Phalloidin 633 and DAPI staining. 200 µl of cellularised TIPS microsphere suspension prepared in 2.3.6 was transferred into a well of an 8 well chambered coverglass. 4 wells were filled from separate cellularized samples. The spent media was removed from each well and the samples were washed with 200 µl DPBS. The cellularized microspheres were fixed by adding 200 µl of 4% paraformaldehyde solution for 10 minutes at room temperature (20°C). The fixative was discarded, and each well was washed with 200 µl of DPBS, for a total of 3 times. 200 µl of 0.1% Triton (T8787, Sigma-Aldrich) was added to each well and the samples were left to incubate at room temperature (20°C) for 5 minutes. The permeabilization solution was discarded and each well was washed with 200 µl of DPBS, for a total of 3 times. A 1% BSA solution was prepared by dissolving 1 g of bovine serum albumin in 100 ml DPBS. 200 µl of 1% BSA solution was added to each well and the samples were blocked for 30 minutes at room temperature (20°C). The Phalloidin stain was prepared by adding 975 µl of DPBS and 25 µl of Phalloidin 568 (A12380, Thermo Fisher) to 1.5 ml microcentrifuge tube. 200 µl of Phalloidin stain was added to each well and the samples were incubated for 30 minutes in the dark at room temperature (20°C). The Phalloidin solution was discarded, and each well was washed with 200 µl of DPBS, for a total of 3 times. The DAPI stain was prepared by adding 1 drop of NucBlue™ Fixed Cell Stain (R37606, Thermo Fisher) in 1 ml of DPBS. 200 µl of NucBlue™ solution was to each well in the last wash step and incubated for 5 minutes. Each well was filled with 200 µl of DPBS, the plate was wrapped in parafilm and stored at 4°C in the dark, for confocal imaging on the next day.

2.3.6.1.2.3 Pluripotent markers
The presence of pluripotent markers SOX2 and TRA-1-60 was assessed using pluripotent stem cell 4-marker immunocytochemistry kit (A24881, Thermo Fisher). The spent media was removed and 200 µl of fixative solution (A24344, Thermo Fisher) was added to each well. The fixative solution was left to incubate at room temperature (20°C) for 15 minutes and was then discarded. 200 µl of permeabilization solution (A24878, Thermo Fisher) was added to each well and left to incubate at room temperature (20°C) for 15 minutes. The solution was then discarded. 200 µl of blocking solution (A24353, Thermo Fisher) was added to each
well and left to incubate at room temperature (20°C) for 30 minutes. 2 µl of anti-SOX2 (host: rat) primary antibody (A24759, Thermo Fisher) and 2 µl of anti-TRA-1-60 (host: mouse IgM) primary antibody (A24868, Thermo Fisher) were added directly to the blocking solution in each well, mixed gently and incubated at 4 °C for 3 hours. The solution was discarded and 200 µl of wash buffer (A24348, Thermo Fisher) was added to each well for 2 minutes. The wash procedure was repeated for a total of 3 times. 200 µl of blocking solution was added to each well along with 0.8 µl of Alexa Fluor™ 488 donkey anti-rat (A24876, Thermo Fisher) and 0.8 µl Alexa Fluor™ 594 goat anti-mouse IgG2a (A24872, Thermo Fisher) secondary antibodies. The solution was left to incubate for 1 hour at room temperature (20°C). The solution was discarded and 200 µl of wash buffer was added to each well for 2 minutes. The wash procedure was repeated for a total of 3 times. The DAPI stain was prepared by adding 1 drop of NucBlue™ Fixed Cell Stain in 1 ml of DPBS. 200 µl of NucBlue™ solution was to each well in the last wash step and incubated for 5 minutes. Each well was filled with 200 µl of DPBS, the plate was wrapped in parafilm and stored at 4°C in the dark, for confocal imaging on the next day.

2.3.6.1.2.4 Cardiac Markers
The spent media was removed and 200 µl affymetrix paraformaldehyde solution, 4% PFA was added to each well. The fixative solution was left to incubate at room temperature (20°C) for 15 minutes. The solution was discarded the samples were washed 3 times with 500 µl DPBS. The wash solution was then discarded. 200 µl of permeabilization solution, 0.5% Triton X 100 (306324N, BDH Lab supplies) in DPBS, was added to each well and left to incubate at room temperature (20°C) for 15 minutes. The solution was then discarded. 200 µl of blocking solution, 3% BSA, 10% goat serum (16210-064, Gibco) in 0.5% Triton X 100-PBS filtered, was added to each well and left to incubate at room temperature (20°C) for 60 minutes. The solution was discarded and a block solution of 3% BSA in 0.5% Triton X 100-PBS filtered, was made for primary antibody incubation. The cells were stained with anti-cardiac Troponin T antibody(Ab45932, Abcam) and anti-Nkx2.5 antibody [2E1] (Ab91196, Abcam), at 1:400 and 1:200 respectively in 3% BSA blocking solution. The cells were incubated overnight at 4 °C. The solution was discarded, and the samples were washed with 200 µl of DPBS, for a total of 3 washes. 200 µl of 3% BSA in 0.5% Triton X 100-PBS blocking solution was added to each well along with
Alexa Fluor™ 488 (A11008, Thermo Fisher) and Alexa Fluor™ 594 (A21125, Thermo Fisher) secondary antibodies at 1:1000 dilution. The solution was left to incubate for 1 hour at room temperature (20°C). The solution was discarded and 200 µl of DPBS was added to each well. The wash procedure was repeated for a total of 3 times. The DAPI stain was prepared by adding 1 drop of NucBlue™ Fixed Cell Stain in 1 ml of DPBS. 200 µl of NucBlue™ solution was to each well in the last wash step and incubated for 5 minutes. Each well was filled with 200 µl of DPBS, the plate was wrapped in parafilm and stored at 4°C in the dark, for confocal imaging on the next day.

2.3.6.2 Migration and Differentiation of iPSC attached on TIPS microspheres
To evaluate the effect attaching iPSC to the TIPS microspheres on their pluripotent phenotype, iPSC were migrated off the TIPS microspheres and onto a VTN-N coated 8 well chambered slide for evaluation of pluripotency. Two 8 well chambered glass slides was coated with 200 µl of 5 µg/ml VTN-N at room temperature (20°C) for 1 hour. The coating solution was discarded and 200 µl of cellularised TIPS microsphere suspension prepared in 2.3.6 was transferred into each well of the chambered slide. 4 wells were filled from separate cellularized samples and duplicated in the other chambered glass slide. The spent media was removed from all wells and replaced with 200 µl of fresh Essential 8 media. Both chambered glass slides were returned to a 37 °C 5% CO₂ incubator for 48 hours. Migration off the TIPS microsphere and onto the chambered glass slide was monitored using a light microscope.

After 48 hours of migration, the cells in the wells of the first chambered glass slide were fixed, permeabilized and stained for pluripotent markers Sox2 and TRA-1-60 as described in section 2.3.6.1.2.3.

The cells in the second chambered glass slide were differentiated into iPSC-CM as described in section 2.3.3.1, up to 21 days and using volumes of 200 µl.

2.3.6.3 Flow cytometry
Flow cytometry was used to assess the cellular phenotype of iPSC and iPSC-CM. Prior to flow cytometry, cell populations isolated from Tryple digestion were washed with DPBS, transferred to a 96-well V-bottom plate (612V96, Thermo Scientific) and kept on ice for the length of the staining protocol.
Samples were analysed on a BD LSR Fortessa I machine. All flow cytometry data was analysed using FlowJo software (Tree Star Inc.).

2.3.6.3.1.1 Cellular staining
To measure cell viability, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (L10119, Thermo Fisher) was used. The lyophilised dye was reconstituted in 50 µl of DMSO. 100 µl of viability dye and extracellular antibodies were added to the cells according to the dilutions in Table 2.1, and incubated for 30 minutes on ice in the dark. The cells were washed DPBS and fixed with 100 µl of 4% PFA solution for 10 minutes at room temperature (20°C) in the dark. The fixative was removed, and the cells were washed twice with DPBS. The samples were permeabilised by washing the cells twice with 100 µl in 1X intracellular staining permeabilization wash buffer (421002, Biolegend). Cells were resuspended in 100 µl of intracellular buffer to which intracellular antibodies were added as detailed in Table 2.1. The samples were stained 4°C for 30 minutes in the dark. The samples were washed twice with DPBS and resuspended in 300 µl of DPBS in FACS tubes (352054, Falcon) ready for analysis. Gates were set based on fluorescence minus one (FMO) stains. Compensation controls were based on single-stained samples for each antibody in the panel and used to create a fluorochrome compensation matrix.
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Table 2.1: Flow Cytometry Antibodies

2.3.6.3.2 Fluorescence-activated cell sorting
BD FACS Aria II was used for fluorescent activated cell sorting (FACS) of GFP+ iPSC. Gates were set based on eGFP negative matched sample. Gating strategy illustrated in section 7.2.1.

2.3.6.4 Quantitative polymerase chain reaction (qPCR)
qPCR was performed to assess pluripotency and differentiation related gene expression from iPSC attached on TIPS microspheres:

2.3.6.4.1 Design of oligonucleotide primers for qPCR
Oligonucleotide primers were designed using Primer Blast, to produce a PCR product size of 70-150 base pairs, predesigned to span or flank introns, anneal at 60°C, <55% GC content and synthesised on a 25 molar scale by Thermo Fisher. Primers were reconstituted with nuclease free dH₂O to a concentration of 100 μM.
Primer efficiency was checked by producing a standard curve of Ct value vs. cDNA concentration per primer pair. cDNA dilutions were prepared: neat, 1:2, 1:4, 1:8, 1:16, 1:32 and blank. Reactions were set in duplicates and qPCR was run as described in section 2.3.6.4.6. Primer efficiency was calculated by plotting Ct values vs. template quantity. The reaction efficiency was calculated by the slope of the best fit line, where 2 represents an efficient reaction (appendix Figure 9.20 – Figure 9.24). Additionally, specificity was tested by amplicon melt curve analysis, by observing for a single peak to confirm specific primer annealing.

2.3.6.4.2 Sample preparation and trilineage differentiation

Control P7 iPSC samples were cultured in VTN-N coated 6 well tissue culture plates (140675, Thermo Scientific) as previously described in section 2.3.2, and harvested after reaching 70-80% colony confluence. Microsphere interaction was investigated by attaching 5×10^5 P7 iPSC to 20 mg of 2% 7507 TIPS microspheres coated with 5 µg/ml of VTN-N, overnight as described in section 2.3.4.1.

Trilineage differentiated cells were obtained from P8 iPSC cultured in 6 well plates at 30-40% culture confluence. The differentiation strategies are graphically summarised in Figure 2.4.
iPSC were differentiated into endoderm progenitor cells using the method described by Ishikawa et al. Endoderm differentiation media was made of Advanced RPMI 1640 media (12633012, Thermo Fisher) supplemented with 1% antibiotic antimycotic solution (A5955, Sigma-Aldrich), 1% Gluatamax (35050061, Thermo Fisher) and 0.2% FBS (10500064, Life Technologies). Differentiation towards endoderm lineage was performed by replacing supplemented E8 media with 2 ml of endoderm differentiation media supplemented with 50 ng/ml activin A (120-4P, Peprotech) and
5 µM CHIR-99021 (1046-5MG, Sigma Aldrich) for 24 hours, followed by a 24-hour treatment with the same medium without CHIR-99021.

Ectoderm differentiation was performed by the method described by Tchieu et al. Ectoderm basal differentiation media was made of 8.2 ml DMEM (41966-029, Gibco), 1.5 ml knockout serum replacement (10828028, Thermo Fisher), 100 µl antibiotic antimycotic solution (A5955, Sigma-Aldrich), 100 µl Glutamax, 100 µl MEM non-essential amino acids (11140050, Thermo Scientific) and 10 µl 2-mercaptoethanol (21985023, Thermo Fisher). Differentiation towards ectoderm lineage was performed by replacing supplemented E8 media with 2 ml of ectoderm differentiation media supplemented with 500 nM LDN193189 (1066208-5MG, Biogems) and 10 µM SB431542 (3014193-1MG, Biogems) for 24 h. The media was replaced with 2ml fresh differentiation media supplemented with LDN193189 and SB431542 for an additional 24 hours.

Mesoderm differentiation was performed as described in section 2.3.3.1. In summary, supplemented E8 media was replaced with 2 ml of Media A for 48 hours.

2.3.6.4.3 RNA extraction
Following the sample preparation described above, the controls samples were detached and harvested into 1.5 ml collection tubes, washed with 1 ml DPBS and a cell pellet was collected. iPSC attached to the TIPS microspheres were washed three times with 1 ml of DPBS, transferred into 1.5 ml collection tubes and the supernatant was discarded. Samples were stored on ice for the length of the extraction.

The workstation was decontaminated of RNA by spraying all tools and work surfaces with 70% ethanol and wiped dry. Filtered pipette tips were used to minimise sample contamination.

RNA extraction was performed using the RNeasy Mini Kit (Qiagen). β-mercaptoethanol was added to Buffer RLT before use, at a concentration of 10 µl β-ME per 1 ml Buffer RLT. The pelleted cells were disrupted by the addition of 350 µl buffer RLT and vortexed at 2000 RPM until the pellet was loosened. The lysates were loaded into a QIAshredder homogenizer spin column (79654, Qiagen) held in a 2 ml RNase free collection tube. The samples were centrifuged for 2 minutes at
maximum speed (13’000 RPM). The column was discarded, 350 µl of 70% cold ethanol was added to the samples and thoroughly mixed by pipetting. 700 µl of sample was transferred into a RNeasy spin column held in a 2 ml RNase free collection tube and spun for 15 seconds at maximum speed. The flow through was discarded. 700 µl of Buffer RW1 was added to the RNeasy spin column to wash the membrane. The sample was spun for 15 seconds at maximum speed and the flow through was discarded. Buffer RPE was reconstituted with 4 times as much 99% ethanol. 500 µL of buffer RPE was added to the spin column and centrifuged for 15 seconds at maximum speed. The flow through was discarded. An additional 500 µL of buffer RPE was added to the spin column and centrifuged for 2 minutes at full speed to remove excess ethanol from the column. The spin column was placed into a new 1.5 ml RNase free collection tube and 30 µL RNase-free water was pipetted directly onto the column membrane with care taken to not touch the sides of the column. The column was spun for 1 minute at full speed to elute the RNA. The column was discarded, and the samples were kept on ice. RNA concentration of the flow through was measured using Nanodrop 8000 UV Visible Spectrophotometer (Thermo Scientific).

2.3.6.4.4 RNA concentration and quality control
Prior to measurement, the Nanodrop spectrophotometer was cleaned with a dry microfiber tissue (07-301-002, ThermoFisher) The NanoDrop software was opened, and RNA analysis selected. The system was calibrated using 1 µL of RNAse free dH2O per well. Once calibration was completed, the RNAse free water was wiped away with a microfiber tissue. 1 µl per sample was pipetted onto separate wells of the stage, the lid was closed, and measurements were acquired. RNA quality and concentration were determined by the provided 260/280 ratio RNA concentration (ng/µl). RNA samples were kept on ice and converted immediately into cDNA or stored at -80°C.
2.3.6.4.5 Reverse transcription of RNA to cDNA synthesis

The extracted RNA was reverse transcribed into cDNA using Quantabio qScript cDNA Supermix (733-1177, VWR). All samples and reagents were kept on ice to avoid RNA degradation. Frozen RNA samples were thawed on ice. 1 µg of RNA in 16 µl of nuclease free dH₂O was transferred into a MicroAmp Fast 0.1 ml 8-Tube Strip (4358293, Thermo). 4 µl of 5x Quantabio qScript cDNA Supermix was added per reaction to a total volume of 20 µl. The samples were vortexed at 1000 RPM and pulsed. The mixed samples were incubated in a MJ Research PTC 200/225 tetrad thermal cycler at:

- 25°C, 5 minutes
- 42°C, 30 minutes
- 85°C, 5 minutes
- Hold at 4°C

The samples were diluted 1:4 with nuclease free dH₂O for PCR reaction and stored at -20°C.

2.3.6.4.6 Relative qPCR to evaluate pluripotency and differentiation related gene expression from iPSC attached on TIPS microspheres

qPCR was used to amplify the full coding sequences of the target genes listed in Table 2.2, to detect and quantify the expression of the genes. The reagents and samples were thawed and kept on ice. 8µM primer pair working solutions were made (from 100 µM primer stocks) for each target gene, containing 8 µl of forward primer, 8 µl of reverse primer and 84 µl of nuclease free dH₂O. 5 µl 2x Brilliant II SYBR Green QPCR (600882, Agilent), 3 µl nuclease free dH₂O, 1 µl primer mix and 1 µl cDNA were mixed and added to a well in a MircoAmp Optical 96-well reaction plate (10411785, Fisher Scientific). Reactions were set up in duplicates and two blank control wells were included. This was repeated with all samples and target genes. The plates were sealed and centrifuged at 600 G for 30 seconds. Gene expression measurement was assessed using an Eppendorf Mastercycler ep Gradient S sequence detection system. The thermal profile was set to run:
<table>
<thead>
<tr>
<th>Cycles</th>
<th>Duration of cycle</th>
<th>Temperature</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>3 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>40</td>
<td>5 seconds</td>
<td>95°C</td>
</tr>
<tr>
<td></td>
<td>15 seconds</td>
<td>60°C</td>
</tr>
</tbody>
</table>

At the end of the cycling, the samples were held at 4°C. Prior to data analysis, the duplicate values were checked to be within 0.5 of each other. The geometric mean of Ct duplicates was taken. Gene of interest expression was normalised to the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ΔCt was calculated as following:

\[\Delta Ct = \text{mean housekeeping Ct} - \text{mean target gene Ct}.\]

Relative gene expression was calculated in absolute values of \(2^{-\Delta Ct}\).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession ID</th>
<th>Primer type</th>
<th>Sequence (5’-3’)</th>
<th>Product length</th>
<th>Start</th>
<th>Stop</th>
<th>Melting temperature</th>
<th>GC%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOX2</td>
<td>NM_003106.4</td>
<td>Forward</td>
<td>GCTACAGCATGATGAGGACCA</td>
<td>135</td>
<td>945</td>
<td>966</td>
<td>62.96</td>
<td>54.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>TCTCGGAGCTGTGATGAGGTT</td>
<td>1079</td>
<td>966</td>
<td>997</td>
<td>63.96</td>
<td>54.55</td>
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<td>POU5F1B</td>
<td>NM_001159542.2</td>
<td>Forward</td>
<td>TTCGGATTTGCCTCTCTCGC</td>
<td>98</td>
<td>60</td>
<td>79</td>
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<td>55</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GGAAGCTTAGCCAGGTCAGA</td>
<td>157</td>
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<td>157</td>
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<td>55</td>
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<td>NANOG</td>
<td>NM_024865</td>
<td>Forward</td>
<td>CTCCAACATCTGAACCTCACG</td>
<td>115</td>
<td>569</td>
<td>590</td>
<td>60.94</td>
<td>54.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CGTCACACATTGCTATCTTTCG</td>
<td>683</td>
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<td>683</td>
<td>60.24</td>
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<tr>
<td>PODXL</td>
<td>NM_005397</td>
<td>Forward</td>
<td>CCTGAACCTCACAGGAACACC</td>
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<td></td>
<td></td>
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<td>62.95</td>
<td>54.55</td>
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<td>Pluripotency</td>
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<td>ALDH1A0</td>
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<td>Reverse</td>
<td>CAACAGCATTGTCCAAGTCGG</td>
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<td>GAGGGCCAAGACGAAGACATC</td>
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<tr>
<td></td>
<td></td>
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<td>CAGATCAGTCTACGCACAC</td>
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<td>PAX6</td>
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<td>DCX</td>
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<td>60.91</td>
<td>57.89</td>
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<tr>
<td></td>
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<td>Reverse</td>
<td>GAACAGACATAGCTTCCCCTTC</td>
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<td>Ectoderm</td>
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<td>NM_001134</td>
<td>Forward</td>
<td>TGAGCACTGTGGCAGAGGAG</td>
<td>96</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>TTGTGTGACAGAGTGCTGTGTA</td>
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<td>GATA1</td>
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<td>777</td>
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<td>52.38</td>
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<td>Reverse</td>
<td>CTGCCGGTTATGCTGACACTA</td>
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<td>TCGACGCCCTTGAGATCACT</td>
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<td>1031</td>
<td>60.98</td>
<td>57.89</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>CTGGGAGACCGGAACATGC</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>52.17</td>
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<tr>
<td></td>
<td></td>
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<td>1782</td>
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<td>DCN</td>
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<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>GTCCTTTCAGGCTAGCTGCACT</td>
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<td></td>
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<td>1346</td>
<td>1325</td>
<td>1346</td>
<td>64</td>
<td>54.55</td>
</tr>
</tbody>
</table>
Table 2.2: qPCR Primers. Oligonucleotide primers were designed using Primer Blast, to produce a PCR product size of 70-150 base pairs, predesigned to span or flank introns, anneal at 60°C, <55% GC content and synthesised on a 25 molar scale by Thermo Fisher.
2.3.6.5 Characterising cardiac contractions

2.3.6.5.1 Sample preparation

The experiments were designed and effected with the help of Dr. Patrizia Camelliti, University of Surrey.

iPSC-CM were purified as described in section 2.3.3.3.2. The contractions of iPSC-CM attached to TIPS microspheres versus iPSC-CM in monolayer attached to tissue culture vessels was compared.

For monolayer attachment, 35 mm plastic dishes (150460, Thermo Fisher) and 10 mm diameter glass coverslip 35 mm dishes (P35G-1.5-10-C, MatTek) were coated with either 5 µg/ml VTN or 50 µg/ml fibronectin overnight at 37°C. The following day, the coating was aspirated from the coated 35 mm dishes, and 150 µL containing 1×10^5 purified iPSC-CM suspension was added to the central coverslip, or the middle of the dish. Dishes were incubated for 24 hours at 37°C, 5% CO₂ to allow cell attachment. After 24 hours, the dishes were carefully washed with pre-warmed (37°C) DPBS to remove cell debris. DPBS was removed and replaced with 1.5 ml pre-warmed to 37°C EB 2% media. Purified iPSC-CMs were incubated at 37°C, 5% CO₂, with media replaced every 2 days. Evidence of spontaneously beating iPSC-CMs was seen from day 2 post-isolation, and cells were used for optical mapping between day 7 and 10 post-isolation.

Suspension iPSC-CM attached to TIPS microspheres were transferred to 35 mm plastic dishes for optical mapping.

2.3.6.5.2 Spontaneous contractions

Spontaneous contractions were recorded using a brightfield microscope. Because cell contractions are temperature sensitive, all the samples were removed from 37°C and allowed to stand at room temperature (20°C) for 1 minute prior to recording. Spontaneous contractions were quantified for 5 minutes at room temperature (20°C), after 5 minutes the samples were returned to the incubator for 30 minutes prior to further counts. For each independent biological sample, 4 different regions were recorded at 60 frames per second (fps) for 30 seconds.
2.3.6.5.3 Optical mapping

Samples were loaded with Ca\(^{2+}\) dye Fluo-4AM (F14201, Thermo Fisher) (4 µM plus 1 mM Probenecid (P36400, Thermo Fisher) in DMEM for 20 min followed by 20 min de-esterification in fresh DMEM). iPSC-CM were superfused with Normal Tyrode’s solution (140 mM NaCl, 4.5 mM KCl, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), pH 7.4) pre-warmed at 37°C. Optical mapping was performed with a Photometrics Evolve 512 EMCCD camera (Photometrics) mounted on a custom macroscope (Cairn Research) equipped with a water-immersion 20x objective, appropriate excitation/emission filters, and 470 nm light-emitting diode illumination. Data was acquired using the recording software WinFluor (Cairn Research). Calcium transient recordings were performed at 200 fps, from an 820 × 820 µm area. The acquired data was analysed using OPTIQ software (Cairn Research) and filtered using a Gaussian spatial filter (radius 2 pixels) before relevant parameters were extracted (Figure 2.5). 5 regions of interest were analysed for each biological replicate (n=3-4 independent biological replicates).

![Figure 2.5: Analysis of calcium transient parameters using OPTIQ software.](image)
2.3.7 Anoikis
2.3.7.1 Sample preparation
P8 iPSC and iPSC-CM were cultured as described in sections 2.3.2 and 2.3.3. Cellularised TIPS microsphere samples were prepared as described in section 2.3.4. Suspension samples were lifted using Tryple or cardiomyocyte dissociation solution as described in section 0 and resuspended in a 6-well low bind plate for 1, 2, 4, 8 or 24 hours. Positive control samples were treated with 2 ml of complete E8 or cardiomyocyte maintenance media supplemented with 10 µM staurosporine (SM97-1, Cambridge Bioscience) for 24 hours or 100 µM etoposide (E1383-25MG, Sigma-Aldrich) for 4 hours.

2.3.7.2 Analysis of protein expression by Western Blot
2.3.7.2.1 Protein isolation
Following the sample preparation described in section 2.3.7.1, the suspension samples and microsphere samples were transferred from the low-binding plates into 1.5 ml microfuge tubes and centrifuged at 200 G for 5 minutes. 6-well plates containing the negative controls and 1.5 ml tubes containing the suspension samples were washed with DPBS. DPBS was discarded, and the 6-well plates were snap frozen on dry ice. The samples in the microfuge tubes were centrifuged, the diluent was discarded while maintaining the pellet, and the samples were also placed on dry ice. The microfuge samples were snap frozen on dry ice and kept overnight at -80°C. Proteins were extracted using a lysis buffer composed of Radioimmunoprecipitation assay (RIPA) buffer (R0278, Sigma-Aldrich) complimented with protease and phosphatase inhibitor (A32961, Thermo Scientific). Snap frozen samples were placed on ice and kept on ice for the length of the extraction. 100-200 µl of lysis buffer was added to each sample and the samples were placed on a rocker for 10 minutes. Negative control samples on plates were harvested using a cell scraper and whole cell lysates were transferred into 1.5 ml microfuge tubes. The samples were centrifuged at full speed for 10 minutes at 4°C, to obtain the soluble protein fraction. The eluted protein was transferred into a new microfuge tube kept on ice. The total protein concentration was quantified using the Pierce Bicinchoninic (BCA) assay (23225, Thermo Scientific).
Bovine serum albumin standards ranging from 0-2000 µg/ml were prepared from the supplier provided ampule, according to manufacturer’s instructions. 25 µl of each standard and unknown sample replicate was pipetted into a 96-well plate. 200 µl of working reagent composed of 50 parts reagent A : 1 part reagent B, was added to each well. The plate was mixed on a plate shaker for 30 seconds and incubated for 30 minutes at 37°C. After 30 minutes, the plate was cooled to room temperature (20°C) and absorbance was measured at 562 nm on a Versamax plate reader. A standard curve was generated by plotting the average blank corrected 562 nm measurements for each BSA standard vs. its known concentration, and this was used to determine the protein concentration of each sample.

Protein concentration was standardised across samples to 30 µg per reaction, by diluting the eluted protein in dH₂O. If the protein concentration was lower than the expected standard, the sample was not diluted. The diluted supernatant was then mixed with 10 µl 4X Bolt LDS Sample Buffer (B0007, Thermo) and 4 µl 10X Bolt Sample Reducing Agent (B0004, Thermo), to give samples of equal volume and protein content. The samples were boiled at 95°C for 5 minutes to denature the proteins and immediately processed for Western blot analysis or stored at -20°C.

2.3.7.2.2 Western blot analysis
Boiled samples and 15 µl of pre-stained protein ladder (PL00001, Protein-Tech) were loaded into the wells of Bolt 4 to 12% Bis-Tris Mini Protein Gel (NW04120BOX, Thermo) submerged into 1:20 diluted 20X Bolt MOPS SDS Running Buffer (B0001, Thermo). The proteins were separated by polycrylamide gel electrophoresis at 200 Volts for 35 minutes. The separated proteins were then electro-transferred onto polyvinylidene fluoride (PVDF) membranes (LC2002, Thermo Scientific). The PVDF membrane was activated by immersion in methanol for 3 minutes, followed by rehydration in dH₂O and a final wash in Bolt transfer buffer (BT00061, Thermo Scientific). The proteins were transferred from the gel onto the PVDF membrane at 35 Volts for 75 minutes. The protein-loaded membranes were blocked with a blocking solution, composed of 5% (w/v) non-fat dried milk in 1:10 diluted 10x Tris Buffered Saline with Tween® 20 (TBST) (12498S, Cell Signalling Technology), for 1 hour at room temperature (20°C) on a rocking platform. The membranes were incubated with primary antibodies overnight at 4°C on a rocking platform. Antibodies were diluted in blocking solutions and the dilutions as described in
Table 2.3. Following primary incubation, the membranes were washed with 1:10
diluted 10x TBST for 10 minutes on a rocking platform, for a total of 3 washes. After
the final wash, the membranes were incubated with horseradish peroxidase (HRP)-
conjugated secondary antibodies diluted in blocking solution, for 1 hour at room
temperature (20°C) on a rocking platform. Following secondary incubation, the
membranes were washed with TBST for 10 minutes on a rocking platform, for a total
of 3 washes. The membranes were covered with Pierce™ ECL Western Blotting
Substrate (32209, Thermo Scientific) for 1 minute. Excess ECL substrate was
removed and the membranes were placed in autoradiography cassettes and
exposed to autoradiography films (34090, Thermo Scientific) in a dark room.

The developed films were scanned at 600 dpi and densitometry analysis was
performed using FIJI software. The quantified protein bands were normalised against
the loading control GAPDH and the protein of interest expression was displayed as
an intensity ratio relative to the loading control.

<table>
<thead>
<tr>
<th>Type</th>
<th>Antibody</th>
<th>Species raised in</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Dilution</th>
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<td>Mouse</td>
<td>9668S</td>
<td>Cell Signalling</td>
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<td>SA00001-2</td>
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<td>Mouse</td>
<td>60004-1-Ig</td>
<td>Protein-Tech</td>
<td>1:10’000</td>
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</table>

Table 2.3: Western blotting antibodies
2.3.7.3 Analysis of cell viability markers by flow cytometry

Cell viability markers were assessed using an Apoptosis/ Necrosis Assay Kit (ab176750, Abcam). Samples were prepared as described in section 2.3.6.1 and cellularised microspheres were decellularized prior to staining, as described in section 2.3.4.3. Suspension samples were collected into 1.5 ml microfuge and centrifuged at 500 x g for 5 minutes in a cold centrifuge. The supernatant was discarded, and the samples were resuspended in 200 µl Assay buffer, 2 µl of Apopxin Deep Red Indicator, 1 µl of Nuclear Green and 1 µl of CytoCalcein 450 stock solutions were added to each sample. The samples were incubated for 30 minutes at room temperature (20°C) in the dark. 300 µl of Assay Buffer was added to each sample prior to flow cytometry analysis. The samples were analysed on a BD LSR Fortessa I machine as described in section 2.3.6.3.

2.4 Delivering TIPS microspheres in vivo

2.4.1 Ethics

All animal studies were approved by the University College London Biological Services Ethical Review Committee and performed with UK Home Office approval (PPL 70/8709 and PPL PP1692884). Animal work conformed to the UK Animals (Scientific Procedures) Act, 1986 and Directive 2010/63/EU of the European Parliament. Signs of stress and the general wellbeing of the animals were observed throughout the study by monitoring of body weight and behaviour.

2.4.2 Implantation of acellular-TIPS microspheres

2.4.2.1 Intramyocardial injections

15 female Sprague Dawley rats 200-250 g (Charles River) were anesthetised in an anaesthesia chamber with 1.5% – 2.5% isoflurane (PRI001325-EA, SAS) in 1.5 - 2 L/min oxygen flow. Each rat was placed in a supine position on the surgery table and connected using a face mask to a continuous flow of isoflurane and oxygen. A 2×4 cm patch was shaved on the left section of the ribcage, above the heart, with a hair removal cream (BodyCurv Bikini and Underarm Hair Removal Cream for Sensitive Skin, Veet). The animal and anaesthetic face mask were transferred and secured to the ultrasound table. Cardiac assessment ultrasound scans prior to microsphere administration were collected using FujiFilm Visualsonics Vevo2100. These included
long axis scan, M Mode measurement of the long axis scan, short axis scan and M
Mode measurement of the short axis scan.

2.4.2.1.1 Delivery of TIPS microspheres
6 rats underwent ultrasound-guided intramyocardial injections of 3 boluses
containing 50 µl of, 20mg 2% 7507 TIPS microspheres suspended in 1 ml 60%
GRANUGEL®, into the basal, mid, and apical region of the left ventricle. A further 3
rats received 3 x 50 µl injections of the microspheres into the left ventricular cavity,
as a worst outcome control and to evaluate the risk of the therapy producing an
embolism. Following the procedure, the rats were placed in heated recovery
chambers for 1 hour, transferred back into their cages and monitored for 7 days. At
day 7, the rats were anesthetised in an anaesthesia chamber with 1.5% – 2.5%
isoflurane in 1.5 - 2 L/min oxygen flow, transferred to the ultrasound table, secured
using tape and cardiac function was reassessed using the ultrasound. Mean ejection
fraction, fractional shortening, left-ventricular mass and left-ventricular volume were
calculated using M-Mode acquisition measurements pre-, post- and 7 days after
injection. To assess the effect of injection on cardiac function, EF of pre-injection,
post-injection and 14 days post-injection were analysed using cardiac package Vevo
lab software version 3.1.1 (Visualsonics, Canada).

At the end of the experiment, the animals were sacrificed using Schedule 1 approved
methods of heart removal and confirmation of death by cervical dislocation. The
heart, lung, liver, and kidney were dissected out, washed with 5 ml of PBS and were
fixed in a 7 ml collection container containing 4% PFA.

2.4.2.1.2 Delivery of BaSO₄ loaded TIPS microspheres
3 rats underwent ultrasound-guided intramyocardial injections of 3 boluses
containing 50 µl of, 46.87 mg of wetted 20% BaSO₄ 2% 7507 TIPS microspheres
suspended in 1 ml 60% GRANUGEL®, into the basal, mid, and apical region of the
left ventricle. A further 3 rats received 3 x 50 µl injections of, 46.87 mg of wetted 20%
BaSO₄ 2% 7507 TIPS microspheres suspended in 500 µl 60% GRANUGEL®, into
the basal, mid, and apical region of the left ventricle. Following the procedure, the
rats were placed in heated recovery chambers for 1 hour, transferred back into their
cages and monitored for 6 days. At day 6, the rats were anesthetised in an
anaesthesia chamber with 1.5% – 2.5% isoflurane in 1.5 - 2 L/min oxygen flow,
transferred to the ultrasound table, secured using tape and cardiac function was reassessed using the ultrasound. Mean ejection fraction, fractional shortening, left-ventricular mass, and left-ventricular volume were calculated using M-Mode acquisition measurements pre-, post- and 6 days after injection. The animals were sacrificed using Schedule 1 approved methods of cervical dislocation and the organs were fixed by cardiac perfusion fixation. The hearts were exposed, the pleural cavity was opened and 20 ml potassium chloride in saline (30 mM KCl, 0.9% w/v (154 mM NaCl) saline) was injected into the left ventricle. Once the eluate drained from the coronary sinus, 20 ml 4% PFA was injected into the left ventricle for whole body perfusion until fixation tremors were visible. The heart, lung, liver, kidney and brain were dissected out, and were fixed in a 7 ml collection container containing 4% PFA overnight and embedded in paraffin.

2.4.2.2 Hindlimb implantation
3 female C57BL/6B mice, 26 weeks old, (Charles River) were anaesthetized as described in section 2.4.2.1. The animals received hindlimb injections of 50 µl of, 46.87 mg of wetted 20% BaSO₄ 2% 7507 TIPS <250 µm microspheres suspended in 1 ml 60% GRANUGEL®, into the right flank and 50 µl of, 46.87 mg of wetted 20% BaSO₄ 2% 7507 TIPS 250-425 µm microspheres suspended in 1 ml 60% GRANUGEL®, into the left flank. Following the injections, the mice were placed in heated recovery chambers for 1 hour, transferred back into their cages and monitored for 7 days. After 7 days, the animals were sacrificed using Schedule 1 approved methods of heart removal and confirmation of death by cervical dislocation.

2.4.3 Implantation of cellularised-TIPS microspheres
The mice were 4 weeks old male immunodeficiency/gamma (NSG) mice (Charles River Laboratories). All in vivo imaging experiments were performed under isoflurane anaesthesia (1.5% – 2.5% isoflurane in 1.5 - 2 L/min oxygen flow).

2.4.3.1 Material preparation
Transduced iPSC were expanded for 3 passages and differentiated into iPSC-CM as described in section 2.3.3.1. On day 18 of differentiation, the cells were purified as described in section 2.3.3.3.2.
6.0 x10^5 cells were attached to 20 mg of hydrophilized 2% 7507 <250 TIPS microspheres as described in section 2.3.4.2. After attachment the cells were resuspended in 500 µl solution of 60% GRANUGEL as described in section 2.1.5. Cell suspension injections were prepared by resuspending 6.0 x10^5 transduced iPSC-CM in 500 µl solution of 60% GRANUGEL. The materials were prepared under aseptic conditions and used within 1 hour of preparation.

2.4.3.2 Intramyocardial injections
Under anaesthesia, mice were positioned in supine position on the ultrasound platform and the limbs were secured with tape. The hair from the chest area was removed by hair removal cream. The ultrasound platform was rotated 45° clockwise to have the apex of the heart facing towards the injection mount. MicroscanTM transducer MS-550D was placed onto the chest and both short and long axis view of the heart was acquired before and after injection using B mode for visualisation and M mode for cardiac function analysis. 7 mice received cell suspension injections and 6 mice received cellularised microsphere injections. Three boluses of 15 µl were injected slowly into the top, mid and apical region of the left ventricle of the heart using a 1 ml syringe with a 23 gauge needle. The successful injection was visualised by the presence of hyperechogenic contrast in the left ventricular cavity. The needle was slowly withdrawn from the body and both short and long axis views of the heart was acquired again. The animals were recovered from anaesthesia and the signs of stress or trauma were observed. Ultrasound evaluation of cardiac function was also performed at the end of the experiment, 14 days post-injection and prior to termination.

At the end of the experiment, the mice were sacrificed by cervical dislocation, and the organs were fixed by cardiac perfusion fixation. Specifically, the chest cavity was opened, and the heart was exposed and the femoral vein/artery was cut. The pleural cavity was opened and the heart reperfused by injection of 2 ml potassium chloride in saline, into the left ventricle. Once the eluate drained from the coronary sinus, 10-20 ml 4% PFA was injected into the left ventricle until fixation tremors were visible. The heart, lung, liver, kidney and brain were dissected out, and fixed in a 7 ml collection container containing 4% PFA overnight and embedded in paraffin.
2.4.4 Bioluminescent Imaging (BLI)
BLI imaging was performed on IVIS Lumina III (In vivo Imaging System, PerkinElmer). For *in vitro* BLI, transduced iPSC from section 2.3.2.1 and subsequently differentiated eGFP+ Luciferase+ iPSC-CM were plated in 96-well plates at concentrations of $8 \times 10^2$ - $5 \times 10^3$ cells per well, in triplicates. After seeding, 5 $\mu$l of 300 $\mu$g/ml D-luciferin was added to each well and thoroughly mixed. Sequential BLI images were acquired 5 minutes after luciferin injection using auto exposure time with 0.5 minutes delay between two consecutive acquisitions. A circular region of interest (ROI) was placed over each well on the first image and subsequently pasted over every new image acquired, up to 1 hour after the addition of luciferin.

Mice from section 2.4.3.2 were anaesthetised and *in vivo* BLI was performed at 1 hour, days 1, 2, 4, 7 and 14 after injection. The mice were injected intraperitoneally with 75 mg/kg D-luciferin (Promega) in 200 $\mu$l of PBS. Sequential BLI images were acquired 5 minutes after luciferin injection using auto exposure time with 0.5 minutes delay between two consecutive acquisitions. A circular ROI was placed over the chest on the first image and subsequently pasted over every new image acquired until all ROIs reached their maximum intensity. Representative ROIs can be found in the appendix (Figure 9.40). 30 minutes after luciferin injection, an image was acquired using a 10-minute exposure time.

The total signal in the ROI was quantified as total flux (photons/s) by using Living Image software (PerkinElmer). Representative images were presented using radiance (the number of photons per second that leave a square centimetre of tissue and radiate into a solid angle of one steradian (sr) = $\frac{\text{photons}}{\text{sec/cm}^2\text{sr}}$) as colour scale.

2.4.5 Fluorescent imaging of ICG loaded TIPS microspheres
Fluorescent images of the TIPS microspheres loaded with ICG were acquired on an IVIS Lumina III at wavelengths 745/820 nm. A circular ROI was placed over the bottom of the polystyrene containers on the first image and subsequently pasted over every new image acquired. The total signal in the ROI was presented using radiance as colour scale using Living Image software.

2.4.6 Computed tomography imaging of BaSO$_4$ loaded TIPS microspheres
Computed tomography (CT) images of TIPS microspheres loaded with BaSO$_4$ were acquired using a PET-CT scanner (Nanoscan, Mediso) with 720 projections at 50
kVp, 170 ms exposure time, and medium zoom. X-ray projections were reconstructed to give CT images with 69 µm isotropic voxel resolution using Interview Fusion software (Mediso). Data were analysed using Vivoquant software (inviCro), with ROIs drawn out manually then thresholded to remove the contribution from air space. Histograms were then produced showing the distribution of signal intensities in Hounsfield units (HU) across voxels in the region of interest, as well as mean HU and SEM.

2.4.7 Evaluation of BaSO$_4$ TIPS microsphere implantation and distribution

2.4.7.1 In vivo whole animal CT
CT images of the mice from section 2.4.2.2 were acquired to monitor the retention of microsphere contrast signal at day 0, 1, 4 and 7 post-implantation. The animals were anesthetized as described in section 2.4.2.1 and laid in a supine position in the PET-CT scanner. CT images were acquired, reconstructed, and analysed as described in section 2.4.6. Following the scan, the mice were placed in heated recovery chambers for 30 minutes and transferred back to their cages.

CT images of the rats from section 2.4.2.1.2 were acquired to monitor microsphere distribution at day 0, 1, 4 and 6 post-implantation. The animals were anesthetized as described in section 2.4.2.1 and laid in a supine position in the PET-CT scanner. CT images were acquired with 720 projections at 50 kVp, 170 ms exposure time, and maximum field of view. Following the scan, the rats were placed in heated recovery chambers for 30 minutes and transferred back to their cages. The CT images were reconstructed and analysed as described in section 2.4.6. 3D ROIs were drawn manually around the heart, based on CT soft-tissue contrast, and bones were segmented using CT signal thresholding.

2.4.7.2 Ex vivo organ CT
CT images of the organs dissected in section 2.4.2.1.2 were acquired immediately after dissection. The organs were dabbed dry from any liquid and CT images were acquired using the PET-CT scanner, with 720 projections at 50 kVp, 170 ms exposure time, and maximum zoom. The images were reconstructed analysed as described in section 2.4.6. 3D ROIs were drawn manually around the heart, lung, liver, kidney and brain, based on CT soft-tissue contrast.
2.4.7.3 Ex vivo heart micro-CT

Micro-CT images were acquired by May Zaw Thin at the Francis Crick Institute.

2.4.8 Histological analysis of tissues

Glass slides were coated with 3-triethoxysilylpropylamine (TESPA), which provides a positive charge that facilitates the attachment of tissue sections. Glass microscope slides (631-1551, VWR) were washed in acetone (20065.362, VWR) for 5 minutes, transferred in a solution of 2% TESPA (440140, Sigma-Aldrich) for 5 minutes and washed twice with dH₂O for 5 minutes. The slides were left to dry at room temperature (20°C) for 18-24 hours.

After fixing, the tissues were cut into 3 horizontal panes and placed into a histology cassette (18000-130, VWR). The tissues underwent dehydration and paraffin embedding using the following protocol:

1. Cassettes were immersed in 70% ethanol for 1 hour
2. 95% ethanol for 1 hour
3. 100% ethanol for 1 hour
4. Fresh 100% ethanol for 1.5 hours
5. Fresh 100% ethanol 1.5 hours
6. Fresh 100% ethanol for 2 hours
7. Histo-clear (AGR1345, Agar Scientific) for 1 hour
8. Fresh Histo-clear for another 2 hours
9. 58°C Paraplast X-tra wax (P3808, Sigma-Aldrich) for 1 hour
10. Fresh 58°C Paraplast X-tra wax for 1-3 hours

The samples were embedded in 15 mm x 15 mm disposable moulds (3804016, Leica). The moulds were filled up to 5 mm with pre-warmed Paraplast X-Tra wax and the tissue was transferred from the cassette to the mould, ensuring that the longitudinal side of the tissue was facing down. Paraplast X-Tra wax was poured over the tissue, ensuring coverage and the bottom of the cassette was placed on top of the mould. The wax was left to harden at room temperature (20°C), after which, the paraffin block was removed from the mould and the samples were stored at -10°C.

The paraffin blocks containing tissue samples were sectioned using a microtome (1512, Leitz). The block was secured to the microtome and aligned in the vertical
plane. The dial was set to cut 5 µm and consecutive sections of paraffin ribbons were cut. The ribbons were picked up with tweezers and transferred to the surface of a pre-warmed 37 °C water bath. The sections were collected at a 30° angle on TESPA-coated slides. The slides were pressed between filter paper (grade 303, 150 mm, 516-0295, VWR) and saturated in 70% ethanol. The blade was replaced upon signs of bluntness and the blocks were refrozen once they started melting. Slides were dried at room temperature (20°C).

2.4.9 Haematoxylin and eosin staining of tissue sections
Slides containing tissue samples were de-waxed and rehydrated prior to staining using the following protocol:

1. slides were incubated in Histo-clear for 5 minutes, twice
2. slides were blotted for excess Histo-clear
3. slides were incubated in 100% ethanol for 5 minutes
4. transferred to 95% ethanol for 5 minutes
5. transferred to 80% ethanol for 5 minutes
6. washed in dH₂O for 2 minutes, twice
7. slides were blotted to remove excess water
8. immersed in haematoxylin for 3 minutes
9. washed in dH₂O for 5 minutes, twice
10. slides were fast dipped into acid alcohol (1 ml hydrochloric acid and 50 ml 70% ethanol) 20 times
11. washed with dH₂O for 5 minutes
12. slides were blotted to remove excess water
13. 1% eosin stock solution was prepared by dissolving 4 g of eosin Y (E4009, Sigma) in 80 ml dH₂O and 320 ml 95% ethanol
14. a working solution of eosin was prepared by combining 100 ml of eosin Y stock solution with 300 ml 80% ethanol and 2 ml glacial acetic acid (537020, Sigma-Aldrich)
15. slides were dipped in the working solution of eosin for 30 seconds
16. the slides were then de-hydrated by incubation in 95% ethanol for 5 minutes
17. 100% ethanol for 5 minutes
18. Histo-clear for 10 minutes.
Slides were mounted using 0.5 ml DPX mountant (44581, Sigma-Aldrich) and sealed with a glass coverslip (AGL463450, Agar Scientific). The stained sections were imaged using a Nanozoomer 2.0-HT (Hamamatsu 61 Photonics). The images were viewed with NanoZoomer Digital Pathology software (NDP Version 2.7.25).

2.5 Statistical analysis
Data reported was collected from technical and biological replicates with samples sizes indicated in figure legends. Data are presented as mean and, standard error of the mean (±SEM) or standard deviation (±SD). Prior to statistical analysis, the data was first tested for normality through a Shapiro-Wilk test. Statistical significance for difference between experimental groups was determined using a Student’s t-test. Analysis of statistical significance for two groups or more was performed using ANOVA followed by post-hoc test. p values of <0.05 indicated statistical significance and were shown as p ≤0.05 = *, p ≤0.01 = **, p ≤0.001 = *** and p ≤0.0001 = ****. No statistical analysis was performed on experiments with n<3. The data were plotted using GraphPad Prism 8 Software (GraphPad Holdings LLC).
Chapter 3: Selection of PLGA TIPS microsphere for attachment of iPSC

This project proposes the delivery of iPSC-CM on TIPS microspheres for cardiac regenerative therapy. The primary aim of this chapter was to screen different PLGA TIPS microsphere formulations and wetting solutions that would support iPSC attachment. Originally it was sought to select a microsphere formulation that would enable the highest attachment of iPSC, and the attached iPSC differentiated into iPSC-CM on TIPS microspheres.

3.1 Introduction

3.1.1 Desirable attributes for cell attachment

In the context of this project, for cellularised TIPS microspheres to be clinically relevant, iPSC must be attached and cultured on this microcarrier efficiently and effectively. Specifically, the substrate should enable cell expansion and maintain control of cell differentiation, by retention of pluripotent cell phenotype and cardiac cell phenotype after differentiation. The key attributes for an ideal implantable cellularised substrate were described in section 1.6.2.

Originally, supporting matrices were required as scaffolds for iPSC to adhere and expand\(^{250}\). These matrices were coated with feeder cells to maximise cellular adhesion and growth\(^{250}\). The most commonly used feeder cells to support iPSC are proliferation-inactivated mouse embryonic fibroblasts. Feeder cell-coated matrices enable iPSC attachment by providing an ECM environment and maintain pluripotency by the release of various proteins critical to pluripotency maintenance, including TGF-\(\beta\)1, activin A, BMP-4, pleiotrophin (heparin-binding growth factor), and more\(^{251}\). However, feeder cells are animal derived and they can increase the risk of pathogen contamination, transfer of animal viruses and/or genetic materials\(^{251}\). Therefore, the microsphere formulation should be an animal component- and feeder cell-free system for the generation of a non-immunogenic therapeutic product, which can be achieved using ECM proteins, conditioned media and synthetic biomaterials.

Another desirable attribute for cell attachment is the surface texture of the substrate. The TIPS microsphere should provide a porous surface area to support the attachment and proliferation of iPSC. Porosity is a highly desirable property for
biomaterials, as it has been shown that mimicking ECM topography can affect cell behaviour. See section 3.1.3.1
Additionally, the TIPS microspheres can provide a dynamic suspension culture system where cells are grown in suspension on the surface of the microspheres under static dynamic conditions. The porous structure allows for movement of nutrients and the suspension system overcomes the induction of the unfavourable gradients of media components seen in static culture conditions\textsuperscript{250}, via enhanced oxygenation, metabolite transport and reduced waste toxicity\textsuperscript{252,253}.

The microspheres should also be biodegradable. This negates the need for removal surgery and the associated complications of an open chest surgery. \textit{In vitro}, by removing the need to enzymatically dissociate F002.1.13 iPSC from VTN-N coated polystyrene microcarriers, by utilising dissolvable microcarriers, Rodrigues \textit{et al.} showed that cells have improved viability, retention of pluripotency and enhanced recovery rates\textsuperscript{250,254}.

3.1.2 Composition of PLGA
PLGA was chosen to fabricate the implantable TIPS microspheres. The utilisation of PLGA as the polymer of choice was discussed in section 1.6.3. Briefly, PLGA is highly tuneable through the control of the polymer’s molecular weight and ratio of PLA and PGA, for the creation of bespoke microspheres\textsuperscript{204}.

In addition to optimising PLGA composition, the tuning of TIPS fabrication parameters provides an additional layer of control over the physicochemical attributes of the substrate.

3.1.3 Physicochemical attributes of the substrate
TIPS fabrication was previously discussed in Section 1.6. Briefly, TIPS was used to screen microsphere formulations, with different PLGA content to support cellular attachment, pluripotent phenotype and delivery. The substrate’s physical properties can affect iPSC behaviour and this has been previously reviewed by Polanco \textit{et al.}\textsuperscript{250}.

The substrate’s physical properties such as, topography, stiffness, hydrophobicity, mechanics and more, can be manipulated through tuning of TIPS fabrication parameters. Physical properties can affect the direction of growth, spread, proliferation, adhesion and differentiation of cells\textsuperscript{255,256}:
3.1.3.1 Topography

It has been shown that when the surface of biomaterials diverge further from native ECM features and mechanics, cell fate is most drastically affected. Hence, it has been suggested that by mimicking the physical characteristics of the ECM on the biomaterial's surface, the attached cell fate can be controlled. Native ECM is porous, rough and it has topological features in the micro- and nano-range. The influence of ECM on cell attachment and fate, has been attributed to the topological features prompting the clustering of cellular integrins. Consequently, this changes the organisation of the cell's actin cytoskeleton and the shape of the cell. Changes in the cell shape influence stem cell differentiation and survival, where rounded cell shapes can trigger apoptosis.

It has been shown that cells will follow the patterns of their culture surface. These patterns can be designed to be suitable to the cell's preferred shape and/or desired application. Li et al. seeded iPSC-CM on thinly aligned PLGA nanofibers to produce tissue like constructs. iPSC-CM aligned and infiltrated the nanofiber sheets, to show an elongated and highly organised structure, similar to in vivo cardiac tissue organisation. The cardiac constructs expressed upregulated cardiac biomarkers and enhanced extracellular recording, compared to iPSC-CM cultured in 2D. The constructs enhanced cardiac function in vitro, by repairing disconnected cardiac tissue and supressing re-entrant arrhythmias in scarred cardiac tissue.

Abadi et al. manipulated the 3D topography of polydimethylsiloxane substrates into cylindrical patterns which resembled native mature CM. Culture of iPSC onto the patterned substrates enhanced differentiation and maturation into iPSC-CM. The physico-mechanical cues generated by the patterned substrate improved the phenotype of iPSC-CM via reorganisation of the cytoskeletal network and regulation of the chromatin structure.
3.1.3.2 Stiffness

The stiffness of the material will also influence cell behaviour. The rigidity of the material will exert a certain amount of strain on the attached cells and impact plasma membrane tension. Plasma membrane tension regulates cellular migration, proliferation, differentiation and survival\textsuperscript{264–266}. Pluripotent stem cells cultured on rigid surfaces will preferentially differentiate into osteocytes\textsuperscript{267}. Whereas, pluripotent stem cells cultured on softer surfaces will preferentially differentiate into adipogenic and chondrogenic lineages\textsuperscript{265,267}.

Hence, a PLGA TIPS microsphere formulation was sought to incorporate the appropriate topography and stiffness to support iPSC and CM attachment, survival and phenotype.

3.1.3.3 Surface Chemistry

TIPS microspheres lack cell-adhesive molecules that are required for the attachment of anchorage-dependent iPSC and iPSC-CM. Cells attach to surfaces through transmembrane cell adhesion proteins called integrins. Without adhesion, anchorage-dependent cells undergo anoikis. Hence, cell-adhesive molecules can be added on microspheres by coating, blending or conjugating cell-adhesive moieties (Figure 3.1)\textsuperscript{193}. 
Figure 3.1: Design modifications that confer cell-adhesive properties to a non-cell-adhesive material. Arg-Gly-Asp peptide (RGD), Tyr-Ile-Gly-Ser-Arg peptide (YIGSR) and Gly-Phe-Hyp-Gly-Glu-Arg peptide (GFOGER). Created with BioRender.

Cell adhesive moieties can be either whole or fragments of ECM proteins, or cell-adhesive polymers, like collagen and fibrin. However, because collagen and fibrin are obtained from xenogenic sources, synthetic peptide fragments that are safer and recognised by cell surface integrins, have been used. These include, Arg-Gly-Asp (RGD) and Tyr-Ile-Gly-Ser-Arg (YIGSR) peptides derived from laminin and Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER) peptide derived from collagen.

A suitable adhesion profile was investigated and selected for the TIPS microspheres to support iPSC culture and phenotype. Vitronectin (VTN-N) coating formulations were investigated, in support of its usage in feeder-free culture of human PSC. VTN-N is a recombinant human protein containing the amino acid fragment 62-478 of human vitronectin, which contains the known RGD cell binding domain. iPSC attach to VTN-N by binding of integrin receptors αvβ1, αvβ3 and αvβ5. Chen et al. reported that VTN-N coating provides a defined surface for cell culture and has been shown to maintain pluripotency and normal growth characteristics in numerous PSC lines.
3.1.4 Aims and Objectives

Aim 1: Determine a suitable TIPS microsphere formulation for iPSC attachment.

Objective 1: Screen different PLGA monomer ratios for the fabrication of TIPS microspheres in order to identify and select a suitable adhesion profile.

Objective 2: Characterise the topography and porosity of the TIPS microsphere formulation using SEM.

Aim 2: Optimise the TIPS microsphere formulation to support iPSC culture and phenotype.

Objective 1: Optimise the substrate’s surface chemistry, namely wetting solutions and adhesive coating parameters to maximise iPSC attachment to the TIPS microspheres.

Objective 2: Characterise the selected TIPS microsphere formulation, including protein adsorption and wetting protocol, to inform the length of microsphere processing time.

Objective 3: Quantify iPSC attachment and proliferation to the TIPS microspheres over 24 hours via preliminary biological characterisation experiments. Namely, microscopic visualisation and automated cell count.
3.2 Results

3.2.1 Selection of TIPS microsphere polymer composition

To establish a suitable TIPS microsphere composition that would enable iPSC attachment, a range of microsphere polymer and coating compositions were investigated.

Three different formulations of 2, 5 and 10 (w/v) %, 7507 PLGA polymer compositions were fabricated. The microspheres were wetted in complete F10 media wetting solution. Complete F10 media wetting solution has been previously used in our group to successfully attach myoblasts to TIPS microspheres and was used in this chapter as an initial wetting solution. iPSC cell attachment was visually followed using light microscopy and quantified at 24 hours, after seeding $2.5 \times 10^5$ iPSC on equal volumes, of 0.1 cm$^3$, of microspheres. Figure 3.2 shows that the lower polymer compositions corresponded to the higher cell attachment. The 2% composition led to significantly higher cell attachment, mean count $12.1 \times 10^4$ cells ($\pm 1.95 \times 10^4$ SEM, n=3, P=0.0268, two-way ANOVA, Tukey’s post-hoc correction), compared with the 10%, mean $4.81 \times 10^4$ cells ($\pm 0.57 \times 10^4$ SEM, n=3). There was no statistical difference between cell attachment to 5% 7507 (mean $9.32 \times 10^4$ cells $\pm 0.96 \times 10^4$ SEM, n=3, P=0.3239 vs. 2% 7507, P=0.1154 vs. 10% 7507), compared to other groups. Hence 2% TIPS microspheres were selected for further characterisation.
Figure 3.2: Quantification of iPSC attachment to 2%, 5% and 10% 7507 TIPS microspheres. 2.5×10^5 P10 iPSC were seeded on equal volumes of 0.1 cm^3 of microspheres, normalised for mass of microspheres, and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) over 24 hours. P=0.0268, two-way ANOVA, Tukey’s post-hoc correction. n=3±SEM.

The surface topography of 2% 7507 TIPS microspheres was assessed using SEM. The micrographs in Figure 3.3 show that 2% 7507 PLGA polymer processed by TIPS, produces microspheres with rough and highly porous topographies. Figure 3.3 B shows that pores ranged in shape and size, and some pores appeared interconnected in Figure 3.3 C.
Figure 3.3: Ultrastructural features of the 2% 7507 TIPS microspheres were assessed using SEM at (A) x400, (B) x1000 and (C) x5000 magnification.

Microsphere samples were prepared at 10 or 20 mg dosages. Quantification of the number of microspheres in a dosage was sought to assess the consistency of the sample preparation. The number of microspheres in 10 mg dry sample was quantified using light microscopy and manual counting. Triplicate counts of 4 separate microsphere production batches were performed. An average of 7603 microspheres were counted in 10 mg of sample, as shown in Figure 3.4. There was no significant difference in the mean microsphere count per production batch (P>0.76, one-way ANOVA, Tukey’s post-hoc correction). The data suggested that 20 mg of dry samples would contain (2×7603 ≈) 15206 microspheres.
The microspheres need to undergo a wetting process, which renders them hydrophilic to enable cell interaction. Addition of cell adhesive moieties was also essential for attachment of iPSC to the TIPS microspheres. Hence, the prospect of coating the microspheres with 5 µg/ml VTN-N directly during or post-wetting was compared. Figure 3.5, shows that direct coating of the microspheres with 5 µg/ml VTN-N in the wetting solution enabled a significantly increased cell attachment (mean count $4.01 \times 10^4 \pm 0.29 \times 10^4$ SEM, n=6, P=0.0001, Unpaired t-test two-tailed), compared to attachment on microspheres coated with 5 µg/ml VTN-N, post-wetting (mean $1.80 \times 10^4 \pm 0.29 \times 10^4$ SEM, n=6). These findings are supported by the representative light microscopy images in Figure 3.6. iPSC attached to TIPS microspheres coated directly during the wetting process and created connecting cellular structures between individual microspheres (Figure 3.6 A). iPSC did not attach nor spread evenly on the surface of microspheres coated post-wetting (Figure 3.6 B), but rather appeared to form clumps near the microspheres.
Figure 3.5: Comparison of direct- versus post-wetting- VTN-N coating of TIPS microspheres. $5 \times 10^5$ P7 iPSC were seeded on equal volumes of 0.1 cm$^3$ of microspheres, matched for mass of microspheres, and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) over 24 hours. Post-wetting coating refers to exposure of the microspheres to 5 µg/ml VTN-N, after wetting. Direct coating refers to exposure of the microspheres to 5 µg/ml VTN-N during wetting. P=0.0001, Unpaired t-test two tailed. n=6±SEM.
Figure 3.6: Representative images of iPSC attached to TIPS microspheres coated (A) directly during the wetting process, or (B) post-wetting. Blue arrows show cell attachment to the microspheres, with formation of cellular connections between microspheres. Red arrows show cellular clumping and uneven attachment of cells to the microspheres. Scale bars represent 100 μm.

The coating composition was refined to determine a suitable TIPS microsphere formulation for iPSC attachment. Previous studies in the group used complete F10 media to wet TIPS microspheres for cell attachment and therefore this solution was used as a starting composition. Figure 3.7 shows that iPSC attached to TIPS microspheres wetted with a complete F10 media solution (mean $3.48 \times 10^5 \pm 0.17 \times 10^5$ SEM, n=6). Supplementing the complete F10 media wetting solution with VTN-N increased the number of iPSC attached to the microsphere, despite the increase not being significant (mean $4.01 \times 10^5 \pm 0.29 \times 10^5$ SEM, n=6). However, wetting solutions consisting of FBS supplemented complete F10 media were not used for further experiments because FBS has been contraindicated for iPSC cell culture. A simplified wetting solution consisting of HBSS and 5 μg/ml VTN-N was investigated and compared with a control solution containing HBSS and 5 μg/ml albumin. HBSS wetting solution supplemented with 5 μg/ml VTN-N enabled the attachment of iPSC overnight (mean $4.67 \times 10^5 \pm 0.35 \times 10^5$ SEM, n=5), at a quantity comparable to the
matched complete F10 media solution supplemented with 5 µg/ml VTN-N (mean $4.01 \times 10^5 \pm 0.29 \times 10^5$ SEM, $n=6$), and significantly higher than complete F10 media solution (mean $3.48 \times 10^5 \pm 0.17 \times 10^5$ SEM, $n=5$, $P=0.0173$, one-way ANOVA, Tukey’s post-hoc correction). A negative control, of HBSS and 5 µg/ml albumin wetting solution, shows that iPSC attachment was negligible and significantly lower than all wetting solutions (mean $0.04 \times 10^5 \pm 0.02 \times 10^5$ SEM, $n=4$, $P=<0.0001$, one-way ANOVA, Tukey’s post-hoc correction). These findings were supported by the representative light microscopy images in Figure 3.8. Majority of iPSC did not attach to the negative control and clumped together (Figure 3.8 A). Seeded iPSC attached to microspheres wetted with HBSS and 5 µg/ml VTN-N (Figure 3.8 B). Therefore, the HBSS and VTN-N wetting solution was carried forward for further experiments.

**Figure 3.7: Selection of wetting solution for the hydrophilisation of TIPS microspheres.** 5×10^5 P6 iPSC were seeded on 20 mg of microspheres wetted with either HBSS and 5 µg/ml albumin (negative control), HBSS and 5 µg/ml VTN-N, complete F10 media and 5 µg/ml VTN-N and complete F10 media (positive control). iPSC were attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. *$P=0.0173$, ****$P=<0.0001$, one-way ANOVA, Tukey’s post-hoc correction. $n=4-6\pm$SEM.
Figure 3.8: Representative images of iPSC attached to TIPS microspheres coated with (A) HBSS and 5 µg/ml albumin, (B) HBSS and 5 µg/ml VTN-N, (C) complete F10 media and 5 µg/ml VTN-N, and (D) complete F10 media. Red arrows show cellular clumping and uneven attachment of cells to the microspheres. Blue arrows show cell attachment to the microspheres, with formation of cellular connections between microspheres. Scale bars represent 100 µm.
The protein coating composition was further refined by assessing the impact VTN-N concentration on iPSC attachment to the microspheres. Microspheres were wetted with a range of 0-20 µg/ml VTN-N in HBSS wetting solutions. Figure 3.9 shows that coating the microspheres in a 5 µg/ml VTN-N HBSS wetting solution enabled the highest attachment of iPSC after 24 hours of seeding (mean count 4.70×10^5 ±0.28×10^5 SEM, n=6, P=<0.0001, two-way ANOVA, Tukey’s post-hoc correction), compared to the negative control. Lower numbers of cells attached to TIPS microspheres with coating concentrations lower than 5 µg/ml, mean cell count of 0.15×10^5 (±0.008×10^5 SEM, n=6) and 0.42×10^5 (±0.12×10^5 SEM, n=6) for 0 and 2.5 µg/ml VTN-N respectively. Interestingly, at coating concentrations higher than 5 µg/ml VTN-N, the number of cells attached to the microspheres was consistently lower at mean count of 1.26-1.39×10^5 cells (±0.05-0.23×10^5 SEM, n=6). Light microscopy images in Figure 3.10 suggest that at higher protein coating concentrations (>5 µg/ml VTN-N), the cells did not evenly attach to the surface of the microspheres, but preferentially clumped together.

**Optimisation of VTN-N coating for iPSC attachment**

![Graph showing cell count comparison](image)

**Figure 3.9:** Comparison of VTN-N protein coating concentration from 0-20 µg/ml for iPSC attachment. 5×10^5 P6 iPSC were seeded on 20 mg of microspheres wetted with HBSS and 0-20 µg/ml VTN-N, and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. **** P=<0.0001, *** P=0.003, P value matched to 0 µg/ml VTN-N negative control. two-way ANOVA, Tukey’s post-hoc correction. n=6±SEM.
Figure 3.10: Representative images of iPSC attached to TIPS microspheres coated with (A) 5 µg/ml, (B) 10 µg/ml, (C) 15 µg/ml and (D) 20 µg/ml VTN-N. Red arrows show cellular clumping and uneven attachment of cells to the microspheres. Scale bars represent 100 µm.
Overall, the data presented in section 3.2.1 shows the development of a novel formulation for the attachment of episomal iPSC to TIPS microspheres. The experimental strategy used for the selection of the microsphere formulation is summarised in Figure 3.11. The 2% 7507 TIPS microsphere formulation, sieved to <250 µm and wetted in 5 µg/ml VTN-N HBSS solution was carried forward. The microsphere size range was selected from needle gauge compatibility, which was investigated in Chapter 4. The selected wetting formulation is also the first example of an animal component-free wetting solution used for TIPS microspheres.
Figure 3.11: Experimental strategy utilised for the selection of the TIPS microsphere formulation that would enable the highest iPSC attachment. Selected parameters shown in green boxes.
3.2.2 Quantification of protein adsorption on TIPS microspheres

During the selection of a wetting formulation in section 3.2.1, it was noted that TIPS microspheres wetted with complete F10 media were sinking faster than microspheres wetted with HBSS and VTN-N. The sinking of microspheres was used as an indicator of wetting as previously described by Oh and Lee\textsuperscript{274,275}.

The length of the wetting process was investigated to inform on microsphere processing time. Firstly, the impact of protein adsorption on TIPS microsphere wetting speed was investigated. A BCA assay was used to quantify the protein adsorption on TIPS microspheres coated with 5 µg/ml VTN-N and 5-20% FBS (matching the content of complete F10 media). The assay quantifies the protein dependent reduction of Cu\(^{+2}\) ions from the copper (II) sulphate to Cu\(^{+1}\) in an alkaline solution and production of purple colour by bicinchoninic acid. Figure 3.12 shows the total protein adsorption to the surface of the microspheres ranged from 100-300 µg/ml depending on the coating composition. The total amount of protein adsorbed to the surface of the microspheres did not appear to be dependent on the concentration of protein in the wetting solution. However, the concentration of protein in the wetting solution affected the rate of microsphere sinking (images in appendix Figure 9.4 - Figure 9.7). The rate of sinking was investigated to assess the length of microsphere processing time, and ultimately processing for \textit{in vivo} delivery.
Figure 3.12: Quantification of protein adsorption on coated TIPS microspheres. BCA assay was used to quantify the protein concentration on the surface of 20 mg 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N and 5-20% FBS for 14 days. Container control refers to the negative control of HBSS with no protein. n=2-3±SEM.

Figure 3.13 A shows that the sinking rate of wetting microspheres was positively correlated to the concentration of FBS in the wetting solution. The higher the FBS concentration in the wetting solution, the faster the sinking rate. Sinking rates differed depending on wetting solution, in FBS wetting solutions, the <250 µm diameter TIPS microspheres sunk within 3 days of wetting, while in VTN-N wetting solution the rate of sinking was slower. Figure 3.13 B shows that majority of <250 µm diameter TIPS microspheres sunk after 14 days of wetting in a 5 µg/ml VTN-N wetting solution. However bigger size ranges did not sink within 14 days of wetting. The corresponding images of sinking microspheres can be found in the appendix (Figure 9.1 and 10.2) and show that TIPS microspheres >250 µm diameter sunk after 4 weeks when exposed to 5 µg/ml VTN-N wetting solution (appendix Figure 9.3).
Figure 3.13: The rate of 2% 75707 microsphere sinking was affected by (A) the concentration of FBS in the wetting solution and (B) the size range of the microspheres. 20 mg 2% 7507 TIPS microspheres were coated with 5 µg/ml VTN-N and 5-40% FBS for 14 days. The percentage of sunk microspheres was visually assessed. n=4-6±SEM.
3.2.3 Characterising the effect of wetting on TIPS microsphere morphology

Section 3.2.2 indicated that microspheres sunk after 4-14 days, and the rate of sinking was dependent on the wetting solution and microsphere size. Next, the effect of wetting on the proportionality of microsphere shrinkage and sinkage was assessed. The assessment further informed the microsphere processing timeframe. The effect of wetting was investigated by assessing the length of the process and the composition of the wetting solution, on the morphology of TIPS microspheres sieved to the size ranges <250, 250-355, 355-425 and 425-500 µm. The microspheres were sieved into 4 different size ranges to determine which diameter range would fit through a suitable needle for delivery into the myocardium of a rodent (chapter 4). Morphology was assessed by microsphere diameter and circularity.

Light microscopy images in Figure 3.14 show that after 14 days of wetting in 5 µg/ml VTN-N wetting solution, the TIPS microspheres shrunk independent of their size range. Figure 3.15 shows that for each size range, median wetted microsphere diameter shrunk by more than half of the pre-wetting diameter. However, the smaller size ranges shrunk faster and the smallest microspheres reached their shrunk diameter by day 1. The larger size ranges shrunk at a slower rate. Additional diameter measurements for day 3, 5 and 7 of wetting can be found in the appendix (Figure 9.8).

Microsphere circularity was not affected by the length of the wetting protocol. All size ranges were unaffected as shown in Figure 3.16. Additional circularity measurements for day 3, 5 and 7 of wetting can be found in the appendix (Figure 9.9).

The effect of three different protein solutions for wetting were investigated: a 20% FBS wetting solution, with high protein content like the complete F10 media wetting solution, a 5 µg/ml VTN-N wetting solution, which enabled the highest attachment of iPSC, and a 2 µg/ml VTN-N wetting solution, as comparative solution with lower protein content. Measurements were taken at day 3 of wetting, due to complete sinking of the microspheres wetted in the 20% FBS solution. Figure 3.17 and Figure 3.18, show that 2 µg/ml VTN-N wetting solution reduced the median circularity of microspheres in all size ranges. Wetting with 20% FBS and 5 µg/ml VTN-N wetting
solutions did not affect microsphere circularity. For diameter measurements, 2 µg/ml and 5 µg/ml VTN-N wetting solutions reduced the median diameter in <250 and 350-425 µm diameter TIPS microspheres, compared to the 20% FBS wetting solution. The median diameter of 250-355 µm microspheres was reduced by 5 µg/ml VTN-N wetting solution but remained comparable when wetting with either 2 µg/ml VTN or 20% FBS wetting solutions. The median diameter of 425-500 µm sieved TIPS microspheres was not affected by the protein concentration in the wetting solution.

Additionally, the sinking inclination of all microspheres was unaffected by their morphology. This was measured by comparing the diameter and circularity of sunk vs. non sunk microspheres at days 3, 5 and 7 of wetting (Figure 3.19).
Figure 3.14: TIPS microspheres of (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm and (D) 425-500 µm diameter (i) are larger pre-wetting and (ii) shrink after 14 days of wetting. Scale bars represent 200 µm.
Figure 3.15: Size range distribution of TIPS microspheres <250 µm (A), 250-355 µm (B), 355-425 µm (C) and 425-500 µm (D) 1 day and 14 days after wetting. The size range of the biggest microspheres shrinks the slowest. n=100
Figure 3.16: Circularity of TIPS microspheres <250 µm (A), 250-355 µm (B), 355-425 µm (C) and 425-500 µm (D) 1 day and 14 days after wetting. n=100
Figure 3.17: The effect of three different protein coatings on the (i) diameter and (ii) circularity of <250 µm (A) and 250-355 µm (B) TIPS microspheres after 3 days of wetting. n=100
Figure 3.18: The effect of three different protein coatings on the (i) diameter and (ii) circularity of 355-425 µm (A) and 425-500 µm (B) TIPS microspheres after 3 days of wetting. n=100
Figure 3.19: Sinking inclination is unaffected by the (i) diameter and (ii) circularity of <250 µm microspheres wetted with 5 µg/ml VTN-N, at day (A) 3, (B) 5 and (C) 7 of wetting. n=100.
3.2.4 Characterising the effect of degradation on TIPS morphology

The effect of simulated *in vivo* degradation of TIPS microspheres was investigated. After wetting and sinking, the microspheres were moved to a fresh solution of HBSS for 3 weeks, at 37 °C under constant rotation at 30 RPM, to simulate the maximum time required for *in vitro* iPSC attachment to the microspheres and differentiation to cardiomyocytes prior to *in vivo* administration. Light microscopy images in Figure 3.20 show that after 3 weeks of degradation, microsphere morphology was not affected. This was supported by quantification of microsphere circularity and diameter in Figure 3.21 and Figure 3.22 respectively. Additional data for 1- and 2-weeks degradation time points are available in the appendix (Figure 9.10 and Figure 9.11).
Figure 3.20: The diameter and circularity of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm and (D) 425-500 µm diameter, remains similar between (i) 14 days of wetting and (iii) after 21 days of dynamic degradation. Scale bars represent 200 µm.
Figure 3.21: 21 days of dynamic degradation does not affect the circularity of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm and (D) 425-500 µm diameter. n=100.
Figure 3.22: 3 weeks of dynamic degradation does not affect the diameter of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm and (D) 425-500 µm diameter. n=100.
3.2.5 Assessing iPSC surface attachment and expansion on TIPS microspheres

From the data in Section 3.2.1, the 2% 7507 TIPS microsphere formulation, sieved to <250 µm and wetted in 5 µg/ml VTN-N HBSS solution was selected for iPSC attachment. The attachment of iPSC to TIPS microspheres was further analysed by confocal microscopy. Confocal micrographs in Figure 3.23 show that iPSC attached to TIPS microspheres within two hours of seeding (A) and expanded to cover the surface of the microspheres (B). The two-hour time point was selected by light microscopy assessment: after 1 hour of seeding the cells appeared attached to the microspheres, but after two hours they appeared attached and spread on the microspheres. After 24 hours of attachment to the TIPS microspheres, iPSC showed integration and total coverage of the surface of the TIPS microspheres. Expansion was due to cell proliferation, which was investigated in chapter 5.

Figure 3.23: Fluorescence microscopy images of iPSC attached to TIPS microspheres stained for nuclei (DAPI blue) and cytoskeleton (phalloidin red), after (A) 2 hours, and (B) 24 hours of seeding. 5×10^5 P7 iPSC were seeded on 20 mg <250 µm 2% 7507 TIPS microspheres coated with 5µg/ml VTN-N and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 2- or 24- hours, fixed and stained. Scale bars represent 100 µm.
From the confocal micrographs at the 2-hour time point, the number of nuclei per microsphere was counted. Figure 3.24 shows that the median cell count was 20 cells per microsphere, minimum was 4, the 25th percentile was 12, 75th percentile was 32 and the maximum cell count per microsphere was 94 cells. The number of cells per microsphere could not be measured at the 24-hours time point, due to cell expansion and microsphere clustering which prevented delineation of separate microspheres.

![Distribution of iPSC count per TIPS microsphere, after 2 hours of seeding.](image)

**Figure 3.24:** Distribution of iPSC count per TIPS microsphere, after 2 hours of seeding. 5×10^5 P7 iPSC were seeded on 20 mg of <250 μm 2% 7507 TIPS microspheres coated with 5μg/ml VTN-N and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 2 hours. After attachment the microspheres were stained for nuclei (DAPI blue) and cytoskeleton (phalloidin red) markers. Z-stack micrographs of the microsphere surface were acquired using confocal microscopy. The number of nuclei on each microsphere was quantified and the number doubled, by assumption that an equal number of cells covered the non-visible part of the microsphere. n=96 microspheres.
3.3 Discussion
The chapter aimed to identify a suitable TIPS microsphere formulation for iPSC attachment. Selection of the formulation would further allow the development of an injectable microsphere formulation to support the delivery of an iPSC-derived cardiac cell therapy to the heart. In addition to this, the wetting parameters measured were used to characterise the fabrication process and processing time.

Selection of 2% 7507 PLGA polymer composition

Selection of the formulation was assessed by quantification of iPSC attachment number. Specifically, the highest iPSC cell count was sought to maximise the number of deliverable cells, along with extensive cell coverage over the surface of the microspheres. A 2% 7507 PLGA polymer composition was selected as it enabled the highest iPSC attachment. The composition corresponds to the polymer formulation with the highest porosity. Surface topography and material stiffness have been shown to affect stem cell fate as described in section 3.1.3, but due to time limitations these effects were not investigated. Hence, the chapter warrants for a more detailed material characterisation, such as characterisation of material topography and stiffness by atomic force microscopy, and ultimately assessment of these parameters on iPSC phenotype. Nonetheless, the effect of the selected TIPS microsphere composition on iPSC phenotype was investigated in chapter 5.

Selection of the direct coating method

The coating and wetting solution were optimised for iPSC attachment, which is a novel cell delivery application for TIPS microspheres. TIPS microspheres are hydrophobic because PLGA is highly hydrophobic. However, a hydrophilic surface is necessary for cellular attachment\(^\text{276}\). By hydrophilising/wetting the TIPS microspheres, the proteins contained in the cell culture medium (such as vitronectin) can adsorb on the TIPS microspheres to promote iPSC attachment by integrin binding. Wetting was achieved by exposing the microspheres to absolute ethanol, as described by Wright \textit{et al.}\(^\text{275}\). To improve the TIPS microspheres compatibility with iPSC culture, the coating composition was optimised by selection of a xeno-free wetting solution composed of HBSS, 11.7 (v/v) % ethanol and a single coating protein, VTN-N. VTN-N is a recombinant human protein routinely used for surface coating of tissue culture plastic for feeder-free culture of iPSC\(^\text{272}\). The recommended
coating concentration for tissue culture plastic is 0.5 μg/cm², and the same concentration of 5 μg/ml was used for coating TIPS microspheres.

Direct coating of TIPS microspheres with 5 μg/ml VTN-N enabled the highest iPSC attachment and further proliferation. Proliferation led to doubling the number of seeded cells within 24 hours. The doubling rate is in accordance with literature. Chatterjee et al. reported a doubling time of 18-20 hours, for iMZ-9 and iMZ-21 iPSC lines grown 2D and coated with attachment factor from Life Technologies. Doubling rate on TIPS microspheres was faster than the results reported by Rodrigues et al., where both Gibco episomal iPSC (same iPSC line used in this project) and F002.1.13 hiPSC lines had doubling rates above 2 days when cultured on Synthemax II coated dissolvable microcarriers.

Coating of the TIPS microspheres post-wetting resulted in a significantly lower number of iPSC attaching, compared to direct coating. With this method of coating, the TIPS microspheres are first exposed to proteins in the complete F10 wetting media and subsequently exposed to the VTN-N coating solution post-wetting. It is possible that other proteins present in the complete F10 wetting media compete for attachment on the surface of the TIPS microspheres during hydrophilisation. Competition might result in lower VTN-N binding to the microspheres post-wetting and subsequent lower iPSC attachment. The protein corona is the protein layer that spontaneously forms around materials when exposed to biological media. Protein binding and exchanging at the corona is governed by the Vroman effect, which originally described how the adsorption of blood proteins to a surface is time-dependent. The first proteins to interact with a biomaterial, are the proteins with the highest diffusion rate and concentrations, but over time these proteins are displaced by proteins with a higher affinity for the surface. During this process, the protein corona evolves to ultimately form a stable protein corona, where proteins with the higher affinity remain strongly, but reversibly, adsorbed to the biomaterial surface. This effect accounts for the hypothesis that only a small fraction of the microsphere surface area/protein corona is available for VTN-N binding after initial adsorption of proteins from complete F10 wetting media. This phenomenon then translates to a lower number of iPSC binding and subsequently attaching to the microspheres.
Selection of the 5 µg/ml VTN-N and HBSS wetting solution

The coating experiments were performed using a hydrophilic solution composed of 10% ethanol and complete F10 media. Initial results showed that attachment of iPSC to TIPS microspheres coated with complete F10 media led a factor increase of 25% of seeded cells, 24 hours post-seeding. Addition of 5 µg/ml VTN-N to the complete F10 media solution slightly improved cell attachment to the surface of TIPS microspheres, to a factor increase of 39% of seeded cells. Complete F10 media contains FBS that has adhesion-promoting properties for cells\textsuperscript{281,282}. The adhesion-promoting properties of serum on tissue culture polystyrene have been attributed to the adsorption of fibronectin and vitronectin\textsuperscript{281,282}. However, media containing FBS and particularly bovine serum albumin, have been known to produce unreliable and irreproducible results, as serum batches varied in capability of maintaining hESC at an undifferentiated stage\textsuperscript{272}. Additionally, FBS has been reported to affect the pluripotency and reprogramming of iPSC. Kwon \textit{et al.} reported that the usage of FBS augmented the reprogramming efficiency for iPSC generation\textsuperscript{283}, but majority of research groups are concerned with the unknown complex mixture of proteins contained in serums that may induce stem cell differentiation\textsuperscript{284}. Because of these concerns, a serum free hydrophilic solution was sought to avoid FBS protein contamination on TIPS microspheres. The wetting solution was optimised to a solution composed of HBSS supplemented with 5 µg/ml VTN-N and 11.7% EtOH. This composition enabled the number of iPSC attached to the surface of the TIPS microspheres to increase at a factor increase of 66% of seeded cells and this was significantly higher than the count of iPSC attached TIPS microspheres wetted with complete F10 media. Attachment was higher than 55% attachment yield (% of original seeded cells) reported by Rodrigues \textit{et al.}, using F002.1.13 hiPSC attached to 5 µg/ml VTN-N coated polystyrene microcarriers\textsuperscript{254}. A negative control of HBSS solution supplemented with 5 µg/ml albumin resulted in 9.5% iPSC attachment, suggesting that VTN-N coating is required to maximise iPSC attachment to the TIPS microspheres.

Interestingly, when validating the optimal VTN-N concentration for coating the microspheres, the concentration of VTN-N in the wetting solution was not positively correlated with iPSC attachment. Highest iPSC attachment was achieved at a coating concentration of 5 µg/ml VTN-N. The coating concentration is consistent with
published literature on VTN-N coated polystyrene microcarriers used for iPSC expansion\textsuperscript{254,285}. At coating concentrations higher than 5 µg/ml VTN-N, the number of cells attached decreased. Light microscopy images in Figure 3.10 suggested that at higher protein coating concentrations (>5 µg/ml VTN-N), the cells did not evenly attach to the surface of the microspheres, but preferentially clumped together. Poor iPSC attachment to TIPS microspheres coated with >5 µg/ml VTN-N could be explained by poor VTN-N binding (and poor RGD orientation for iPSC binding) at higher protein concentration.

VTN-N has been found to enable cell binding once adsorbed to both negative and positively charged substrates. Bernards \textit{et al.} inferred the importance of VTN bioactivity to its conformation, once adsorbed on charged surfaces. Li \textit{et al.} using computational and experimental approaches, confirmed that the conformational changes of VTN during adsorption onto charged monolayers affect cell adhesion \textsuperscript{286}. The study found that cell adhesion was possible on all VTN coated surfaces, but charged monolayers affected the orientation of VTN binding domains and resulted in differences in cell adhesion\textsuperscript{286}. The N-terminal part of VTN is composed of a somatomedin-B domain and a cell receptor binding site, characterised by an Arg-Gly-Asp (RGD) sequence. The RGD sequence is considered the core functional cell binding domain. The accessibility of the RGD motifs dictates cell adhesion to VTN by interaction with cellular integrin receptors, such as αvβ1, αvβ3 and αvβ5. Li \textit{et al.} suggested that different charged surfaces affect the unfolding dynamics of adsorbed VTN, subsequent exposure of the RGD domain, and ultimately affecting cell binding capabilities\textsuperscript{286}. The study made two relevant observations: (1) in neutral hydrophilic surface interaction, destabilised attachment of VTN, by repeated adsorption and desorption, resulted in unsettled RGD orientation and reduced the capability for cell binding\textsuperscript{286}. (2) in neutral hydrophobic surface interactions, VTN molecules were squashed, stacked into high density multilayers, and restraining the RGD binding loops\textsuperscript{286}. These suggestions could explain the results observed at >5 µg/ml VTN protein coating concentrations, where competitive protein binding or VTN layering might have occurred, resulting in poor RGD orientation and subsequently poor iPSC binding.
Characterisation of the wetting protocol

The rate of wetting was investigated to assess the length of microsphere processing time. <250 µm 2% 75075 TIPS microspheres wetted with 5 µg/ml VTN-N required 14 days of wetting, while microspheres wetted with complete F10 media required 3-4 days of wetting to sink. The longer wetting time with VTN-N resulted in longer microsphere processing time and this informed the timeframe for in vivo delivery. Characterisation of the wetting protocol on TIPS microsphere morphology and degradation suggested that microspheres shrunk during wetting, and the rate of shrinking was dependent on the original microsphere size. It is hypothesized that shrinking of microsphere diameter occurred from collapse of internal microsphere porous structures, which could be practically investigated by cryo-electron microscopy analysis of wetted samples. However, due to time limitations this investigation was outside the scope of the project.

Sinking of the microspheres was used to determine the completion of the wetting procedure and the completion of microsphere processing. The rate of sinking was dependent on the original microsphere size. The results align with reported literature. Computational modelling by Fang et al. determined that the kinetics of protein adsorption on polymer surfaces change as a function of time, because the surface layers deform as the proteins adsorb\textsuperscript{287}. The finding supports the observations that microspheres shrink during wetting, but bigger microspheres will shrink slower. Most relevantly, the range and strength of the repulsive interaction on adsorbing proteins increases in relation to polymer molecular weight and surface coverage\textsuperscript{287}. Hence, protein adsorption, shrinking and therefore sinking is slower in microspheres with bigger diameters, as seen in Figure 3.13.
iPSC attached and expanded on the surface of the TIPS microspheres

To characterise iPSC adhesion on the TIPS microspheres, confocal microscopy was used to visualise cellular attachment to the microspheres. After 24 hours of seeding, iPSC covered the whole surface of the microspheres and cellular bridging was seen between iPSC on separate TIPS microspheres. The images suggest that seeded iPSC utilise the full surface area of TIPS microspheres and have the potential to proliferate after attachment. The cell count per microsphere in Figure 3.23 was limited by the inability to visualise half of the microsphere by z-stacking on a confocal micrograph. Hence, the count relied on the assumption of even cell distribution on the non-visible half of the microsphere. Additionally, cell count per microsphere could not be directly measured at the 24-hour time point, due to cell expansion and microsphere clustering which prevented delineation of separate microspheres. This will be explored in proliferation and phenotypic assays in chapter 5 of this thesis.

Overall, the selected wetting formulation composed of HBSS, 11.7% ethanol and a single coating protein, VTN-N, is the first example of an animal component-free wetting solution used for TIPS microspheres. Additionally, assessment of microsphere wetting provided a timeframe of microsphere processing of 14 days. This was used in upcoming chapters to inform the planning of in vitro and in vivo experiments.

This chapter presents the first ever reported attachment of human iPSC to TIPS microspheres, which could provide a platform for iPSC expansion and differentiation into iPSC-derived cell products for different applications.
3.4 Summary

This chapter aimed to screen different PLGA TIPS microsphere formulations and wetting solutions that would support iPSC attachment. The wetting protocol was also characterised to inform the length of microsphere processing time. A graphical summary of Chapter 3 is given in Figure 3.25. The results demonstrated that:

- PLGA polymer composition, wetting solution composition and protein coating were optimised to determine a suitable TIPS microsphere formulation for iPSC attachment. 2% 7507 TIPS microsphere formulation, sieved to <250 µm and wetted in 5 µg/ml VTN-N HBSS solution was selected for further experiments. The wetting formulation is the first example of an animal component-free wetting solution used for TIPS microspheres.
- SEM analysis of the topographical features of 2% 7507 PLGA TIPS microspheres showed that the microspheres exhibit a highly porous and rough surface topography.
- Protein adsorption on the surface of the microspheres was quantified using a BCA assay.
- Characterisation of the wetting protocol on TIPS microsphere morphology and degradation suggested that:
  - Microspheres shrink during wetting, and the rate of shrinking was dependent on the original microsphere size.
  - Protein concentration in the wetting solution, to some extent affected, the circularity, but not the diameter of sinking microspheres.
  - There was no morphological difference between sunk and unsunk microspheres.
  - Simulated in vitro degradation did not affect microsphere morphology.
- First ever reported attachment of human iPSC to TIPS microspheres.
- iPSC attached to the selected microsphere formulation within 2 hours. The cells further expanded to cover the surface of the microspheres after 24 hours of seeding.

The selected formulation was taken forward for assessment of compatibility for minimally invasive delivery to the heart (chapter 4) and cellular compatibility (chapter 5 and 6).
Figure 3.25: Graphical summary of Chapter 3.
Chapter 4: Functionalization of TIPS microspheres for in vivo tracking

4.1 Introduction
For in vivo delivery, the injection of TIPS microspheres should be safe and accurately targeted to a specific location within the myocardium. In vivo tracking of the microspheres would allow confirmation of accurate delivery, persistence at the target site and location of any off target biodistribution. This chapter aimed to determine if this could be achieved using clinically applicable non-invasive imaging techniques.

4.1.1 Desirable parameters for an injectable microsphere formulation
TIPS microspheres were produced to be an injectable therapeutic and compatible with minimally invasive delivery. The following delivery parameters were considered to achieve retention of the TIPS microspheres in the myocardium of the left ventricle:

(1) Suspension formulation
A suspension formulation was sought for delivery of the TIPS microspheres in vivo. Microsphere buoyancy (floating vs. sinking) would affect the repeatability and delivery. To create a suspensive formulation, the hydrocolloidal gel GRANUGEL® was used. GRANUGEL® was selected due to its existing clinical approval in humans for treatment of open wounds.

(2) Animal model
Healthy female Sprague Dawley rats were chosen to be the preclinical animal model for this chapter. Healthy animals were chosen to evaluate preliminary safety parameters of treatment with the selected TIPS microsphere formulation, particularly fatality and side effects. Acellular microspheres were used in these preliminary safety and tracking experiments.

(3) Target site and dosage
Intramyocardial injections can target and localize the therapy to specific regions of the myocardium of the LV. The anterolateral wall was chosen for injection as it is the most commonly affected area in animal models of ischaemic injury and shows the most extensive CM loss. Previous preclinical studies utilizing rat models, delivered between 50 µl to 300 µl of injectable therapeutics, including microspheres and...
cell sheets, via intramyocardial injections. The values informed the dosage range in this study. Chow et al. delivered 3 × 50 μL dosages of hydrogel containing iPSC-CM around the myocardial infarct border zone on the LV. The multiple bolus system enabled the delivery of the hydrogel treatment to the top, mid and apical region of the myocardium. Given that the volume of a rat LV is 600-800 μl, the volume of the injection dose is equal to ¼ to ⅕ of the volume of the target site. This proportion has been shown to be safe and comply with animal welfare guidelines. Hence, 3 × 50 μL dosages were used in this chapter and the maximum number of microspheres loaded in the dosage was quantified.

(4) Delivery device
To inject a precise dosage of the microsphere formulation, 250 μl and 1 ml syringes were used. A suitable needle gauge was investigated, to enable the delivery of the TIPS microspheres effectively and safely. The inner gauge of the needle needed to be wide enough to deliver the suspension formulation of TIPS microspheres without blockages. Additionally, the smallest bevel was sought, to ensure full embedding of the needle into the muscle.

(5) Minimally invasive delivery method
In order to improve cell engraftment in the target site, local cell delivery to the heart was sought. Recent cell therapy trials have described the delivery of biologics to the heart by intracoronary injections, intravenous injections, retrograde coronary venous injections, intrapericardial injections and intramyocardial injections. The delivery routes of cell therapy products to the heart have been thoroughly reviewed elsewhere by Li et al. The aforementioned delivery routes and associated theories of cell loss are summarised in Figure 4.1.
Intravenous, intracoronary and trans endocardial delivery of cell therapies to the heart are generally performed by minimally invasive catheter injections. In contrast, epicardial and intramyocardial injections are normally performed by direct surgical access to the heart through a thoracotomy. The invasive open-chest surgery increases the risk of intervention-related morbidities and limits the clinical application. Portions of the heart, such as the dorsal surface, are not easily visualised and targeted using this method.

For this study, the microspheres were delivered by ultrasound guided transthoracic intramyocardial injections. The novel delivery method for microspheres assisted cell therapy negates the need for a thoracotomy or sternotomy, making the delivery technique minimally invasive and localised to the myocardium. The technique also provides greater flexibility on the timing and sites of injection by ultrasound guidance. Additionally, delivery of the microspheres by ultra-sound guided injections is superior to alternative cell sheet transplantation in the heart. Cell sheet transplantation is an invasive procedure as it requires a thoracotomy for the cell sheet to be placed onto the epicardial surface.

Overall, the above parameters were investigated *in vitro* to achieve localized retention of acellular TIPS microspheres at the target site *in vivo*. Dow *et al.* injected...
neonatal rat cardiac cells in the left ventricular wall of Fischer rats subjected to ischaemia reperfusion injury\textsuperscript{117}. Immediately after administration major organs were explanted for histology and qPCR analysis\textsuperscript{117}. The study found that the injectable therapies in the heart are difficult to retain at the target site and they are lost to the systemic circulation by the pumping action of the heart. The delivered cells were found in off-target sites, in the circulation, lungs, kidney, liver and spleen, where they could not provide direct cardiac replacement therapy\textsuperscript{117}. By maximising localization, cellularised TIPS microspheres are likely to be more effective.

4.1.2 Tracking materials \textit{in vivo}
Tracking of implanted materials can be achieved using a variety of non-invasive imaging modalities. Imaging modalities rely on the interactions between the applied energy format (i.e. light, magnetic, X-ray and sound) and the material and/or tissue. These interactions result in the absorption, scattering and polarization of the emitted energy by the investigated objects (Figure 4.2)\textsuperscript{291}. Imaging modalities can be distinguished by their contrast mechanisms, to practically select the most appropriate modality to differentiate between host and implanted objects\textsuperscript{291}. Imaging modalities can be categorised as acoustic, magnetic, optical, electron, X-ray and nuclear imaging by their contrast mechanism (Figure 4.2)\textsuperscript{291}. Table 4.1 summarizes the properties of each imaging modality.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{contrast_mechanisms.png}
\caption{Contrast mechanisms to enhance imaging capacity. Biomedical imaging modalities rely on the interaction between the applied energy format and the material and tissue. Imaging modalities can be categories by their contract mechanism. Adapted from Zhang & Yao\textsuperscript{291}.}
\end{figure}

Each imaging modality can be used to visualize certain properties of the materials of interest. However, the sensitivity achieved may not be satisfactory to characterize the desired interaction between the material and \textit{in vivo} model. Hence, it may be
necessary to introduce contrast mechanisms, to either the biological or non-biological entities, to make them visible under the imaging modality of interest. This chapter investigates the creation of contrast generating and therefore trackable TIPS microspheres using fluorophores or x-ray contrast agents. Fluorescent and CT tracking have the depth penetration (>1 cm) and 100µm isotropic spatial resolution required to distinguish between endocardial and epicardial injections, as well as to track the localization of implanted microspheres.
<table>
<thead>
<tr>
<th>Imaging modality</th>
<th>Contrast mechanism</th>
<th>Typical spatial resolution</th>
<th>Typical penetration depth</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Representative applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasound</td>
<td>Acoustic reflection (back scattering)</td>
<td>0.3 mm</td>
<td>10 cm</td>
<td>• Non-invasive • High speed • Deep penetration</td>
<td>• Superficial penetration • Low chemical sensitivity • Requires coupling medium</td>
<td>• Mechanics • Flow dynamics • Scaffold cavitation</td>
</tr>
<tr>
<td>Magnetic resonance imaging</td>
<td>Proton magnetization and relaxation</td>
<td>1 mm</td>
<td>50 cm</td>
<td>• Non-invasive • Deep penetration</td>
<td>• Expensive • Slow imaging speed</td>
<td>• Fluid content • Fluid transport</td>
</tr>
<tr>
<td>Multi-photon microscopy</td>
<td>Fluorescent emission, optical scattering</td>
<td>1 μm</td>
<td>1 mm</td>
<td>• Non-invasive • Cellular-level resolution • High chemical sensitivity</td>
<td>• Superficial penetration</td>
<td>• Cell attachment of scaffolds • Gene expression</td>
</tr>
<tr>
<td>Optical coherence tomography</td>
<td>Optical back scattering</td>
<td>10 μm</td>
<td>2 mm</td>
<td>• Non-invasive • Cellular-level resolution • High imaging speed</td>
<td>• Superficial penetration • Low chemical sensitivity</td>
<td>• Vascularization • Cell tracking • Scaffold degradation</td>
</tr>
<tr>
<td>Photoacoustic tomography</td>
<td>Optical absorption</td>
<td>0.1 mm</td>
<td>10 cm</td>
<td>• Non-invasive • High functional and chemical sensitivity • Deep penetration</td>
<td>• Requires coupling medium</td>
<td>• Vascularization • Oxygenation • Cell tracking • Cell-biomaterial interaction</td>
</tr>
<tr>
<td>X-ray imaging</td>
<td>X-ray absorption</td>
<td>0.1 mm</td>
<td>40 cm</td>
<td>• Non-invasive • Deep penetration • High resolution</td>
<td>• Ionizing radiation • Low chemical sensitivity</td>
<td>• Engineered bone • Pore structures</td>
</tr>
<tr>
<td>Positron-emission tomography/ single-photon emission computed tomography</td>
<td>Gamma ray emission</td>
<td>5 mm</td>
<td>50 cm</td>
<td>• Non-invasive • Deep penetration • High molecular imaging</td>
<td>• Low resolution • Radiative labelling</td>
<td>• Cell-biomaterial interaction • Mineralization</td>
</tr>
</tbody>
</table>

Table 4.1: The properties of clinically available imaging modalities. Adapted from Zhang & Yao.²⁹¹.
4.1.3 Aims and Objectives

Aim 1: Determine a suitable delivery formulation for the injection of TIPS microspheres.

Objective 1: Identify a suitable GRANUGEL® delivery formulation that will hold the microspheres in suspension for >3 hours

Objective 2: Quantify the number of the microspheres delivered per treatment.

Aim 2: Demonstrate the safety of delivering TIPS microspheres *in vivo*.

Objective 1: Deliver the TIPS microspheres in the top, mid and basal myocardium of a rat model using ultra-sound guidance to attain localized and retained delivery.

Objective 2: Evaluate the initial safety parameters of delivering acellular TIPS microspheres, including toxicity, side effects and mortality, to rationalise the future use of the cellularised therapeutic in a rodent model.

Objective 3: Justify the need for a trackable composition of TIPS microspheres to improve the monitoring of the therapeutic *in vivo*.

Aim 3: Formulate a composition of TIPS microspheres that can be tracked *in vivo* without causing unwanted side-effects for cell delivery using the microcarriers.

Objective 1: Identify suitable contrast agents compatible with fabrication of TIPS microspheres.

Objective 2: Evaluate the biocompatibility of toxicity of the labelled TIPS microspheres.

Objective 3: Demonstrate the compatibility of labelled TIPS microspheres for localized delivery.
4.2 Results
4.2.1 Selecting an injectable delivery formulation
4.2.1.1 Granugel suspension

To deliver the TIPS microspheres in a uniform suspension and avoid sedimentation in the syringe, a GRANUGEL® dilution that would enable the microspheres to stay in suspension for 3-4 hours was investigated. The time point was chosen to reflect the maximum time required to formulate the cellularised material in vitro, to in vivo administration (based on existing laboratory protocols and facility access). 0.1 cm$^3$ of 2% 7507 wetted TIPS microspheres were overlaid on the surface of 1 ml dilutions of 20-60% (v/v) GRANUGEL®, and time for microspheres sedimentation was measured (Figure 4.3). Dilutions with a higher concentration of GRANUGEL® kept the microspheres in suspension for longer amounts of time. Microspheres suspended in the 40% dilution had completely sedimented after 180 minutes, in 50% dilution sedimented after 240 minutes and in 60% dilution remained in suspension over 4 hours. The 60% dilution was carried forward because of the longer suspension time and ease of injectability. Suspensions of 70% and above GRANUGEL® were more viscous, difficult to inject through a syringe and therefore not investigated.

<table>
<thead>
<tr>
<th>GRANUGEL® dilution</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinking time (min)</td>
<td>5</td>
<td>5</td>
<td>180</td>
<td>240</td>
<td>&gt;240</td>
</tr>
</tbody>
</table>

Figure 4.3: Selection of a GRANUGEL® dilution for the delivery of TIPS microspheres in suspension. 0.1 cm$^3$ of 2% 7507 wetted TIPS microspheres were overlaid on the surface of 1 ml dilutions of 20-60% (v/v) GRANUGEL®, and time for microspheres sedimentation was visually assessed. Blue arrows indicate the location of the sinking TIPS microspheres suspension in the tube. n=1.
4.2.1.2 Needle gauge

To enable minimally invasive, but targeted delivery of the TIPS microspheres into the heart, a 23G needle was selected for administration. The gauge of the needle was the biggest that could be safely used for intramyocardial administration in a rat model, based on previous experience in the Stuckey lab (data not shown). 20 mg of 2% 7507 TIPS microspheres <250 μm diameter were suspended in 1 ml 60% GRANUGEL® dilution. 250 μl of the test solution was loaded into a 250 μl Hamilton syringe fitted with a bevelled 23G needle. The 23G needle permitted the flow of TIPS microspheres sieved to <250 μm diameter without occlusion. The flow of microspheres of different size ranges through a 23G needle was investigated and it was found that 2% 7507 TIPS microspheres <250 and 250-355 μm in diameter could flow through. However, none could pass through a smaller gauge (25G). Data available in the appendix (Figure 9.12 and Figure 9.13).

4.2.1.3 Quantification of the number of TIPS microspheres delivered

As stated in Section 4.1.1, 3 × 50 μl bolus injections were identified as a feasible volume for delivery into the myocardium of rats. The number of TIPS microspheres loaded in each bolus was quantified to estimate the number of TIPS microspheres delivered in vivo using a semi-automated Morphologi G3 particle size and particle shape image analyzer. 250 μl of the test solution was loaded into a 250 μl Hamilton syringe fitted with a 23G needle. 50 μl boluses were injected onto the surface of a microscope slide before being analysed with the Morphologi G3. The data were then manually verified to eliminate image artifacts. Table 4.2 summarises the microspheres count per bolus and dose (a dose was composed of 3 boluses). The quantity of TIPS microspheres delivered by each 50 μl volume was inconsistent, ranging from 352 microspheres to 2275. On average each treatment delivered 4198 microspheres (± 544 SEM, n=4). This could have been due to poor mixing of the GRANUGEL® and microspheres. Additional shape image analysis and respective frequency distribution are available in the appendix (Figure 9.14).
<table>
<thead>
<tr>
<th>Bolus number</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
<th>Dose 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1962</td>
<td>352</td>
<td>1116</td>
<td>1568</td>
</tr>
<tr>
<td>2</td>
<td>1823</td>
<td>1554</td>
<td>1691</td>
<td>542</td>
</tr>
<tr>
<td>3</td>
<td>1952</td>
<td>2275</td>
<td>744</td>
<td>1212</td>
</tr>
<tr>
<td>TOTAL</td>
<td>5737</td>
<td>4181</td>
<td>3551</td>
<td>3322</td>
</tr>
<tr>
<td>Average</td>
<td>4198 ± 544 (13%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Quantification of the number of TIPS microspheres delivered in a dosage. Each dose was composed of 3 boluses of 50 µl, for the total delivery of 150 µl of TIPS microspheres suspended in 60% GRANUGEL® vehicle. n=4±SEM.

4.2.2 Evaluating the feasibility of delivering TIPS microspheres into the myocardium
A rat model was used to assess the feasibility of delivering TIPS microspheres into the myocardium. The experimental set up is summarised in Figure 4.4. 9 female Sprague Dawley rats weighing 200 - 250 g were allocated into two groups to receive ultrasound-guided injections of the TIPS microspheres. Group 1 (n=6) rats received intramyocardial injections (positive control) and Group 2 (n=3) rats received injections in the left-ventricular cavity (negative / worst case scenario control). The expected worst-case scenario was that accidental leakage of the microspheres from the myocardium, through the left ventricular wall, and into the circulation. Given the size of the microspheres (~80 µm diameter after shrinking), leakage into the circulation could lead to systemic embolization causing stroke, hindlimb ischemia, pulmonary embolism, or myocardial infarction. Treatment groups received a pre-injection assessment of cardiac function via ultrasound analysis, followed by ultrasound guided intramyocardial injection or left-ventricular injections and a post-injection assessment scan. The animals were monitored for 6 days and received a follow up cardiac function assessment scan. At this point the animals were sacrificed and histological analysis was performed on the heart, lung, liver and kidney to assess the distribution of the delivered TIPS microspheres.
Figure 4.4: Diagrammatic representation of the delivery protocol for TIPS microspheres into a rat model. Treatment was composed of 3 boluses of 50 µl, for the total delivery of 150 µl of TIPS microspheres suspended in 60% GRANUGEL® vehicle. The cardiac function was assessed pre-injection at day 0, by ultrasound. Animals received injections in either the top, mid or apical region of the LV myocardium (n=6) or LV cavity (n=3). After 6 days of recovery, the cardiac function was re-assessed using ultrasound analysis and the animals were sacrificed for further histological analysis. Created with BioRender.

3 boluses of 50 µl, containing acellular TIPS microspheres were delivered into the basal, med, and apical region of the left ventricle (Figure 4.5 A and B). Likewise, 3 boluses containing the same TIPS microspheres formulation were delivered in the cavity of the left ventricle (Figure 4.5 C and D). Intra-myocardial injections of the TIPS microspheres appeared to result in successful delivery into the myocardial wall, indicated by the presence of hyperenhancement at the site of injection (Figure 4.6).
Figure 4.5: Targeted localization of the TIPS microspheres in the basal, mid and apical section of the left ventricular (A-B) myocardium and (C-D) cavity. Localization shown by (A and C) diagrammatic representation and (B and D) needle localization during the ultrasound-guided procedure. Endocardium represented by yellow dotted line and tip of needle represented by green asterisk. Created with BioRender.

Figure 4.6: Short axis ultrasound scan of the mid-section of a heart post-intramyocardial injection of TIPS microspheres. The red arrow points to hyperenhancement in the myocardial wall beside the left ventricle.
Analysis of cardiac function (Figure 4.7) showed that implantation of the TIPS microspheres in the myocardium or left ventricular cavity did not significantly affect changes in the left ventricular mass nor ejection fraction, immediately following injection and at 7 days post-injection. Additional measurements of heart function were also acquired (Figure 4.8) and are summarised in Table 4.3. There was a trend towards increased left ventricular mass immediately after injection (Figure 4.8 B), which likely reflected TIPS microspheres retention in the myocardium, but this trend did not reach significance.

**Figure 4.7: Assessment of cardiac function following implantation of acellular TIPS microspheres.** Cardiac function was measured by ejection fraction, before and after TIPS microspheres implantation. Two-way ANOVA, Tukey’s post-hoc correction. n=3-6±SEM.

<table>
<thead>
<tr>
<th></th>
<th>1000µl resuspension</th>
<th>500µl resuspension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-injection</td>
<td>Post-injection</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>74.74</td>
<td>69.22</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>44.81</td>
<td>39.99</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>640.58</td>
<td>695.33</td>
</tr>
<tr>
<td>LV volume, at diastole (µl)</td>
<td>244.10</td>
<td>251.38</td>
</tr>
<tr>
<td>LV volume, at systole (µl)</td>
<td>71.63</td>
<td>66.07</td>
</tr>
</tbody>
</table>

**Table 4.3: Summary of cardiac function analysis.** Values are the means reported in Figure 4.8, n=3-6. Cardiac function was measured by ultrasound analysis, before and after acellular TIPS microspheres injections.
Figure 4.8: Changes in (A) fractional shortening, (B) LV mass, LV volume at (C) diastole and (D) systole. Additional measures of cardiac function were measured by ultrasound analysis, before and after TIPS microspheres implantation. Two-way ANOVA, Tukey’s post-hoc correction. n=3-6±SEM.
4.2.3 Formulating trackable TIPS microspheres

4.2.3.1 Indocyanine green (ICG) loading

To enable the real-time localisation of TIPS microspheres *in vivo*, production of trackable microspheres was investigated. Initial attempts explored production of fluorescent TIPS microspheres loaded with ICG, which had comparable morphology to the unloaded microspheres (Figure 4.9). The ICG loaded microspheres showed high porosity but had smaller and unevenly distributed porosity. However, the ICG loaded TIPS microspheres did not fluoresce brightly (Figure 4.10).

![Figure 4.9: Ultrastructural features of ICG loaded TIPS microspheres. SEM images at (A) x400, (B) x1000 and magnification. Scale bar represents 50µm.](image)

4.2.3.2 ICG coating

An alternative production approach was taken to produce trackable microspheres, by coating hydrophilised TIPS microspheres with a layer of ICG. The intensity of the fluorescence signal from control, ICG solutions (Figure 4.11 A-D), wetting solutions (Figure 4.11 E-G) and ICG coated TIPS microspheres was imaged. TIPS microspheres that were hydrophilised and coated with ICG dissolved in ethanol and media had the brightest fluorescent signal. However, the fluorescent signal was below the threshold for *in vivo* tracking.
Figure 4.10: Measurement of fluorescent signal produced by ICG-loaded microspheres. 20mg of ICG loaded TIPS microspheres were placed in a 7 ml tube and the fluorescence was immediately measured using an IVIS Lumina at wavelengths 745/820 nm. Regions of interest (ROI) manually drawn over the location of the microspheres to obtain a total epi-fluorescent signal.
Figure 4.11: Measurement of the fluorescent signal produced by ICG-coated microspheres. Fluorescent images of (A) cell media, (B) dH$_2$O, ICG dissolved in (C) dH$_2$O and (D) ethanol respectively. TIPS microspheres coated with ICG formulations: (E) water solute and ethanol, (F) ethanol solute and water, (G) ethanol solute and F10 formulation.
4.2.3.3 Barium sulphate (BaSO₄) loading

The third approach taken to produce trackable TIPS microspheres involved loading the TIPS microspheres with a contrast agent for CT tracking. BaSO₄ was loaded in the TIPS microspheres during the fabrication process and its signal was measured against control TIPS microspheres without BaSO₄ (Figure 4.12). The TIPS microspheres loaded with 20% BaSO₄ showed contrast above 2000 HU (Figure 4.12 C). This value was above the attenuation factors of soft tissue and bone, which suggested that the microspheres would be distinguishable for tracking in vivo. This composition of TIPS microspheres also showed detectable signal when loaded in the 60% GRANUGEL® delivery vehicle (Figure 4.12 F) and after 7 days of simulated degradation in cell culture medium (Figure 4.12 G).

The morphology of the 20% BaSO₄ loaded microspheres was assessed using SEM (Figure 4.13). Loading of BaSO₄ resulted in the porosity of the TIPS microspheres being retained but led to a change in the surface topography. Surface pores were visible with a regular pore pattern, but the pores were smaller in size. The loading of BaSO₄ on the surface of the TIPS microspheres was visible and confirmed at the higher magnification (Figure 4.13 C). The 20% BaSO₄ loaded TIPS microspheres were carried forward for further in vitro and in vivo characterisation.
Figure 4.12: Contrast signal produced by BaSO₄-loaded microspheres. CT scans of (A) 2%, (B) 6%, and (C) 20% (w/v) BaSO₄ TIPS microspheres. Control (D) TIPS without BaSO₄ and (E) GRANUGEL®. 20% BaSO₄ loaded microspheres in (F) GRANUGEL® and (G) after 7 days of simulated degradation under static conditions.
Figure 4.13: Surface features of 20% w/v BaSO₄ loaded microspheres. SEM images at (A) x400, (B) x1000 and (C) x5000 magnification. Red arrows indicate the presence of BaSO₄ crystals on the surface of the microsphere. Scale bar represents 10µm.

4.2.4 Investigating BaSO₄ loaded TIPS microspheres

4.2.4.1 Physical assessment

Loading of BaSO₄ in the microspheres was further investigated using Energy Dispersive X-Ray Spectroscopy (EDX). The technique, similar to SEM, scans the surface of a sample with a beam of electrons, and the interaction of the sample with electron beam produces X-rays. The X-rays released correspond to the valance electron energy of specific atoms. The characteristic X-rays are collected and the elemental composition of the sample is calculated. Control 7507 TIPS microspheres were analysed and compared to BaSO₄ loaded microspheres. Representative electron images (Figure 4.14 A) and elemental analysis (Figure 4.14 B) of control microspheres indicated the presence of carbon and oxygen on the surface of the control microspheres. Traces of sodium and sulphur were also detected at negligible amounts. EDX analysis in Figure 4.15 confirmed the presence of barium and sulphur in the BaSO₄ loaded microspheres. Figure 4.15 B highlighted the elemental peaks of barium in blue and sulphur in red.

This presents the first example of CT trackable BaSO₄ TIPS microspheres.
Figure 4.14: EDX analysis of control 2\% 7507 250-355 \(\mu\)m TIPS microspheres. (A) Electron image and (B) overlayed EDX image, showing the presence of (i) carbon in red, (ii) oxygen in green and (iii) sodium in blue. (C) Corresponding elemental analysis. Scale bar represents 100 \(\mu\)m.
Figure 4.15: EDX analysis of 2% 7507 20% BaSO₄ 250-355 µm TIPS microspheres. (A) Electron image and (B) overlayed EDX image, showing the presence of (i) carbon in red, (ii) oxygen in green, (iii) sulphur in blue and (iv) barium in pink. (C) Corresponding elemental analysis. Scale bar represents 250 µm.
BaSO₄ loading affected the density of the microspheres (Figure 4.16). The number of microspheres in 10 mg of dry sample was quantified by manually counting dispersed microspheres on a microscope slide. The mean number of microspheres in 10 mg of 2% 7507 TIPS microspheres was 7603 (± 430.1 SEM, n=3) microspheres, while the mean count in 10 mg of 20% BaSO₄ loaded 2% 7507 TIPS microspheres was significantly lower (3245 ± 127.8 SEM, n=3, P=<0.0001, unpaired t-test two tailed).

46.87 mg of 20% BaSO₄ loaded microspheres was used in further experiments to equalize the number of microspheres present in 20 mg of 2% 7507 TIPS microspheres.

**Figure 4.16:** Quantification of TIPS microspheres in 10 mg. Manual count of 3 separate production batches using light microscopy. P=<0.0001, Unpaired t-test two tailed. n=3±SEM.
4.2.4.2 In vitro biocompatibility

The biocompatibility of 20% BaSO$_4$ loaded microspheres was assessed using a lactate dehydrogenase (LDH) assay. LDH is a cytosolic enzyme and the assay measures the release of LDH as indicator of cellular toxicity. Damage to the cellular plasma membrane results in the release of LDH into cell culture medium. Released LDH is measured by a coupled enzymatic reaction. Extracellular LDH catalyses the conversion of lactate into pyruvate via NAD$^+$ reduction to NADH. Diopharase uses NADH to reduce a tetrazolium salt (INT) into a formazan product that can be measured at 490 nm. The amount of red formazan is directly proportional the amount of LDH released in the medium.

Conditioned media was obtained by incubating complete DMEM to either 20 mg of wetted 2% 7507 TIPS microspheres (negative control) or 46.87 mg of 2% 7507 20% BaSO$_4$ TIPS overnight under dynamic conditions. Vehicle control media was composed of unconditioned complete DMEM. L929 were exposed to conditioned or complete media (vehicle control) up to 7 days and LDH release was assessed. LDH release was corrected to percent cytotoxicity to a positive control sample (maximum LDH release). Figure 4.13 shows that addition of vehicle control reduced toxicity by 13.90 % ($\pm$1.26 SEM, n=4) and control 2% 7507 TIPS microspheres conditioned media reduced toxicity by 12.41% ($\pm$1.56 SEM, n=4) after incubation for 1 day.

Conditioned media from 20% BaSO$_4$ loaded microspheres reduced toxicity by 2.77% ($\pm$2.69 SEM, n=4) and this was significantly different to the vehicle control (P=0.0021, matched to vehicle control, two-way ANOVA, Dunnet’s post-hoc correction). Day 3 toxicity from 2% 7507 microspheres was 9.45% ($\pm$3.43 SEM, n=4), higher than the other groups, but this was not significant (p=0.26). At day 7, conditioned media from 20% BaSO$_4$ loaded microsphere produced a significantly higher toxicity (mean 12.00 % $\pm$2.89 SEM, n=4, P=0.0459, matched to vehicle control, two-way ANOVA, Dunnet’s post-hoc correction), compared to vehicle control (mean 5.01%).

Despite the significant difference in toxicity, the 20% BaSO$_4$ loaded microspheres formulation was the best option available and was carried forward to characterisation and short term trackability studies, without microspheres cellularisation. The in vivo
studies with the 20% BaSO₄ loaded microspheres were planned to last 7 days to determine the safety and retention of the delivery method. To note, it was not planned for this formulation to be celllarised as BaSO₄ is not biodegradable and the resulting toxicity might not be tolerated in long-term animal studies.

Figure 4.17: Toxicity of BaSO₄ loaded TIPS microspheres to L929. 1 ml of complete DMEM was added to 20 mg of wetted 2% 7507 TIPS microspheres (negative control) and 46.87 mg of 2% 7507 20% BaSO₄ TIPS. The samples were incubated overnight under dynamic conditions. 100 µl of conditioned media or unconditioned medium (vehicle control) was added to 1×10⁵ L929 cells and cytotoxicity was assessed after 1, 2, 3 and 7 days incubation. Medium was replaced every two days with fresh conditioned medium. ** P=0.0021, * P=0.0459, P values matched to vehicle control. two-way ANOVA, Dunnet’s post-hoc correction. n=4±SEM

The effect of wetting on the morphology of 20% BaSO₄ loaded microspheres was investigated. The 20% BaSO₄ loaded microspheres wetted with 20% FBS were compared to 2% 7507 control microspheres wetted with either 5 µg/ml VTN-N or 20% FBS, as described in chapter 3. Figure 4.18 shows that 20% BaSO₄ loaded microspheres did not shrink at the same rate as control microspheres wetted in either VTN or FBS wetting solutions. The diameter of wetted 20% BaSO₄ loaded microspheres remained similar to the diameter of the pre-wetting sample. Circularity distribution was comparable to the control microspheres. Descriptive statistics for Figure 4.18 are summarised in Figure 4.19.
Figure 4.18: BaSO\textsubscript{4} loaded TIPS microspheres <250 µm do not shrink at the extent as control TIPS microspheres. Changes in (A) diameter and (B) circularity, after 1 day of wetting in either 5 µg/ml VTN-N or 20% FBS. n=100.
Figure 4.19: Descriptive statistics of (A) diameter and (B) circularity of degrading BaSO₄ loaded TIPS microspheres compared to control TIPS microspheres. Statistics to Figure 4.18. n=100.
4.2.4.3 Preliminary *in vivo* trackability

The trackability of BaSO$_4$ loaded microspheres was initially assessed in a mouse model. C57BL/6J mice ($n=3$) received hindlimb injections of $50 \mu l$ 2% 7507 20% BaSO$_4$ TIPS <250 $\mu m$ and 250-355 $\mu m$ in formulations previously described in Section 4.2.1. Hindlimb delivery was performed as a preliminary study due to the ease of administration and localised monitoring of contrast signal over time. A diagrammatic summary of this experiment is included in Figure 4.20. Post-administration, the contrast signal was tracked for 7 days with CT.

Figure 4.20: Diagrammatic representation of the delivery protocol for 20% BaSO$_4$ TIPS microspheres into a mouse hindlimb model. 46.87 mg of 2% 7507 20% BaSO$_4$ TIPS <250 $\mu m$ and 250-355 $\mu m$ were wetted overnight in a solution of HBSS and 5 $\mu g/ml$ VTN. The wetted microspheres were resuspended in 1 ml 60% GRANUGEL® solution. The mice ($n=3$) were anesthetised and received injections containing $50 \mu l$ of <250 BaSO$_4$ microspheres suspension in the right hindlimb and $50 \mu l$ of 250-355 BaSO$_4$ microspheres suspension in the left hindlimb. The persistence of the microspheres localization was assessed at day 0, 1, 4 and 7 post-injection, by whole body CT scan. Created with BioRender.

Whole body CT scans were acquired and the contrast signal from the hindlimbs was quantified using ROIs as shown in the representative scans in Figure 4.21 A. Both groups showed a retention of contrast signal in the hindlimb over the 7-day time course (Figure 4.21 B). 250-355 $\mu m$ BaSO$_4$ microspheres produced a higher mean contrast signal, than 250 $\mu m$ BaSO$_4$ microspheres. This result was expected, as the bigger microspheres contained a higher level of BaSO$_4$ (in total).
Figure 4.21: The signal from implanted BaSO4 microspheres in the hindlimb retains contrast up to 7 days. (A) Example ROI in the (i) sagittal, (ii) coronal and (iii) transverse view used for signal analysis, and (B) the analysed mean contrast signal over 7 days. n=2-3 ± SEM.

Representative images of reconstructed whole-body CT scans are included in Figure 4.22. Reconstructed BaSO4 TIPS microspheres (pink) <250 µm and (blue) 250-425 µm were localised and retained at the injection site in the hindlimb. Overall, these preliminary data suggested that it was feasible to track microspheres retention and justified proceeding with the cardiac delivery experiments.
Figure 4.22: Reconstructed CT scans of the contrast signal emitted by BaSO$_4$ TIPS microspheres (pink) <250 µm and (blue) 250-425 µm, at day (A) 0, (B) 1, (C) 4, and (D) 7 post injection.
4.2.4.4 *In vivo* cardiac trackability

6 female Sprague Dawley 200-250 g were allocated into two groups to receive ultrasound-guided injections of 250 µm 20% BaSO$_4$ loaded TIPS microspheres. The study design is summarised in Figure 4.23. Treatment groups received a pre-injection assessment of cardiac function via ultrasound analysis, followed by ultrasound guided intramyocardial injections (3 boluses of 50 µl) in the top, mid or apical region of the LV myocardium and a post-injection assessment scan. The first group (n=3) received a high dose (46.87 mg wetted microspheres resuspended in 500 µl 60% GRANUGEL® solution) and the second group (n=3) received a low dose (46.87 mg wetted microspheres resuspended in 1000 µl 60% GRANUGEL® solution). The animals were monitored for 6 days, CT scanned on day 0, 1, 4, and 6. On day 6, the cardiac function was re-assessed using ultrasound analysis and the animals were sacrificed. *Ex vivo* CT and histology was performed on the heart, lung, liver and kidney to confirm the presence of embedded 20% BaSO$_4$ loaded TIPS microspheres.
Figure 4.23: Diagrammatic representation of the delivery protocol for 20% BaSO₄ TIPS microspheres into a rat model. 46.87 mg of 2% 7507 20% BaSO₄ TIPS <250 µm were wetted overnight in a solution of HBSS and 5µg/ml VTN. The wetted microspheres were resuspended in 500 µl (high dose) or 1 ml (low dose) 60% GRANUGEL® solution. Treatment was composed of 3 boluses of 50 µl, for the total delivery of 150 µl of TIPS microspheres. The cardiac function was assessed pre-injection at day 0, by ultrasound. Animals received injections in the top, mid or apical region of the LV myocardium. The persistence of microspheres localization was assessed at day 0, 1, 4 and 6 post-injection, by whole body CT scan. On day 6, the cardiac function was re-assessed using ultrasound analysis and the animals were sacrificed. The major organs, including heart, lungs, kidneys, liver and brain, were removed for ex vivo CT scans and further histological analysis. Created with BioRender.

Videos of ultrasound scans pre-, during-, post-injection and 3D high-resolution reconstructions were acquired. The scans showed the needle in the myocardium, delivering the microspheres which produced hyperenhancement in the myocardial wall. The hyperenhancement was retained post-injection as shown in the 3D reconstruction.

Administration of two different dosages of microspheres did not adversely affect cardiac functions, as measured by ejection fraction (Figure 4.24). In the low dose group (1000 µl resuspension), the significantly lowered ejection fraction post-injection (mean 69.22% ±1.50 SEM, n=3, P=0.0419, two-way ANOVA, Tukey’s post-hoc correction) was due to the depression of cardiac contractility from the length of the anaesthesia. At the post-injection time point, the animals had been kept under
anaesthesia for a longer time (30-45 minutes), compared to pre-injection and post-recovery (10-15 minutes).

![Graph showing ejection fraction over time](image)

**Figure 4.24: Assessment of cardiac function following implantation of acellular BaSO₄ TIPS microspheres.** Cardiac function was measured by ejection fraction, before and after microspheres injection. * P=0.0419, two-way ANOVA, Tukey’s post-hoc correction. n=2-3±SEM.

Additional measurements of heart function were also acquired (Figure 4.25) and mean values are summarised in Table 4.3. In the low dose group there was a significantly lowered fractional shortening (Figure 4.25 A) (mean 39.99% ±1.188 SEM, n=3, P=0.0475, two-way ANOVA, Tukey’s post-hoc correction) and difference in LV at systole (Figure 4.25 D) (mean 77.631 µl ± 6.51 SEM, n=4, P=0.0316, two-way ANOVA, Tukey’s post-hoc correction) after injection, but after 6 days recovery the values normalised to the pre-injection measurements.

Whole body CT scans were acquired and the contrast signal from the heart was quantified using ROIs as shown in the representative scans in Figure 4.26. Representative 3D whole body CT scan reconstruction images are available in the appendix (Figure 9.15). Both groups showed a retention of contrast signal in the heart over the 6-day time course (Figure 4.27), suggesting that majority of the microspheres were retained in the target tissue.

Despite the retention of contrast signal, there were differences in contrast signal between animals. The microspheres injected in rat 3 emitted the highest contrast signal despite receiving a low dose. The difference in signal is attributed to the
challenges of the delivery technique; injecting into an area that is 2-3 mm thick under the guidance of ultrasound is difficult, particularly in a beating heart.

<table>
<thead>
<tr>
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<th>1000μl resuspension</th>
<th>500μl resuspension</th>
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<tbody>
<tr>
<td></td>
<td>Pre-injection</td>
<td>Post-injection</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
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<td>69.22</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>44.81</td>
<td>39.99</td>
</tr>
<tr>
<td>LV Mass (mg)</td>
<td>640.58</td>
<td>695.33</td>
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<tr>
<td>LV volume, at diastole (μl)</td>
<td>244.10</td>
<td>251.38</td>
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<tr>
<td>LV volume, at systole (μl)</td>
<td>71.63</td>
<td>66.07</td>
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Table 4.4: Summary of cardiac function analysis. Values are the means reported in Figure 4.25, n=2-3. Cardiac function was measured by ultrasound analysis, before and after BaSO₄ TIPS microspheres injections.
Figure 4.25: Changes in (A) fractional shortening, (B) LV mass, LV volume at (C) diastole and (D) systole. Additional measures of cardiac function were measured by ultrasound analysis, before and after BaSO₄ TIPS microspheres implantation. * P=0.0475 (A), * P=0.0316 (B), two-way ANOVA, Tukey’s post-hoc correction. n=2-3±SEM.
Figure 4.26: The signal from implanted BaSO4 particles retains contrast in the heart. Example ROI in the (A) sagittal, (B) coronal and (C) transverse view used for signal analysis, and derived microspheres in green.
Figure 4.27: Persistence of the contrast signal emitted from the BaSO₄ microspheres injected in the heart. The contrast signal was analysed (A) over a time course of 6 days and (B) compiled per animal. n=4±SEM.

Observations across whole body CT scans showed signs of off-target delivery. Representative scans in Figure 4.28 show microspheres localization outside the heart. *Ex vivo* CT and microCT reconstruction confirmed the exact location of the microspheres in the *ex vivo* heart. Representative images of 3D reconstructions are available in the appendix (Figure 9.16). *Ex vivo* CT of explanted organs (Figure 4.29) also confirmed that a low level of microspheres was visualised in off-target organs including lung, brain and liver, indicating that some material was lost during injection.
Figure 4.28: Missed delivery of BaSO₄ loaded TIPS microspheres. CT scans in the (A) sagittal, (B) coronal and (C) transverse view shows BaSO₄ loaded TIPS microspheres implantation below the heart and above the diaphragm.
Figure 4.29: Visualisation of off target delivery of BaSO$_4$ loaded TIPS microspheres. Representative images of microspheres distribution in the major organs ex vivo.

The uptake from *ex vivo* organs showed patterns of distribution consistent with *in vivo* data. The majority of contrast signal emitted from the microspheres was localised to the heart, followed by lower observation in by the lungs and trace amounts in the kidney and liver (Figure 4.30 A). Volumetric normalisation of the microspheres deposition to the volume of the organ was also consistent with the *in vivo* data (Figure 4.30 B). The constructed and analysed ROIs of *ex vivo* organs are included in the appendix (Figure 9.17 – Figure 9.19).
Figure 4.30: Quantification of off target delivery of BaSO₄ loaded TIPS microspheres. Quantification of (A) contrast signal and (B) % organ volume occupation by the BaSO₄ loaded TIPS microspheres in the major organs *ex vivo*.

An animal from the high dosage group had to be sacrificed post-injection due to poor recovery. Analysis of whole-body CT scans of the sacrificed animal revealed contrast signal emitted from the brain, suggestive of the presence of BaSO₄ in the brain (Figure 4.31).
Figure 4.31: Visualisation of off target delivery of BaSO$_4$ loaded TIPS microspheres to the brain. Whole animal CT scans of (A) control and (B) early sacrifice rat to assess distribution of BaSO$_4$ loaded TIPS microspheres in the brain. (i) sagittal and (ii) coronal view.
Figure 4.32: Quantification of off target delivery of BaSO$_4$ loaded TIPS microspheres to the brain. CT scans of the ex vivo brains of (A) control and (B) early sacrifice rat in (i) sagittal and (ii) coronal view, and (C) quantification of the contrast signal from respective brains. n=1.

The signal from ex vivo brain showed patterns of distribution consistent with in vivo data. The signal of the early sacrifice animal (Figure 4.32 B) was compared to the signal of a control animal (Figure 4.32 A) that had not shown signs of stressed recovery, nor contrast signal in the brain in whole body CT scans. Ex vivo CT scans showed that microspheres were present in the brain of the control animal, suggesting that ex vivo organ analysis is more sensitive to picking up the contrast signal emitted from single microspheres. Quantification of the CT scans showed that the contrast signal emitted from the early sacrifice was 3 times higher than the contrast signal of the control animal (Figure 4.32 C).

To further evaluate the distribution of 20% BaSO$_4$ loaded TIPS microspheres, the explanted organs underwent histological analysis. Interestingly, the microspheres were visible by eye in the ex vivo hearts (Figure 4.33).
Figure 4.33: Visible BaSO$_4$ microspheres in the ex vivo heart. (A-D) 360° whole organ and (E) cross sectional view of an ex vivo heart. Red arrows indicate the presence of visible BaSO$_4$ loaded TIPS microspheres.

Histological sections were stained with H&E. Histology of the explanted hearts confirmed implantation of the BaSO$_4$ loaded TIPS microspheres in the left ventricle (Figure 4.35 - Figure 4.34). Inflammation was not seen immediately after implantation (Figure 4.34), but after 6 days of implantation a significant immune response was visible around the site of microspheres implantation and following the needle track (Figure 4.35 - Figure 4.37). This was consistent with the time scale of foreign body response.
Figure 4.34: H&E stained heart section shows the BaSO₄ loaded TIPS microspheres in the left ventricular wall of a rat 3 hours after administration. (A) Tissue cross section shows the location of the implantation site. (B) higher magnification image indicates the presence of the microspheres in grey. Scale bar represents 100 µm.
Figure 4.35: H&E stained heart section shows the implantation of BaSO₄ loaded TIPS microspheres in the left ventricular wall of a rat 6 days after administration. (A) Tissue cross section shows the location of the implantation site. Yellow arrows in (B) the higher magnification images indicate the presence of the microspheres. 100 µm scale bar.
Figure 4.36: H&E stained heart section shows the implantation of BaSO₄ loaded TIPS microspheres in the left ventricular wall of a rat, and the resulting inflammation following the needle track. (A) Cross sectional view of left ventricular chamber, with needle track shown in red rectangle. (B) Expanded view of the needle track and immune cell infiltrate across the ventricular wall. The yellow arrows indicate the presence of the BaSO₄ loaded TIPS microspheres. Scale bar represents 200 µm.
Figure 4.37: H&E stained heart section shows the implantation of BaSO₄ loaded TIPS microspheres in the left ventricular wall of a rat, and the resulting inflammation following the needle track. (A) Cross sectional view of left ventricular chamber, with needle track shown in red rectangle. (B) Expanded view of the needle track across the ventricular wall, scale bar represents 1 mm. (C) Expanded view shows presence of immune cell infiltrate. Yellow arrows indicate the presence of the BaSO₄ loaded TIPS microspheres. Scale bar represents 250 µm.
Histological analysis of kidney (Figure 4.38) and brain (Figure 4.39) sections showed that a minority of the microspheres are present in off-target blood vessels. This suggests that the microspheres reached off-target organs by the circulation, with evidence of red blood cells present in the microspheres.

Figure 4.38: H&E stained section shows BaSO₄ loaded microspheres in a blood vessel of brain. (A) Tissue cross section shows the location of the blood vessel containing the microsphere. (B) The higher magnification image shows the blood vessel enclosing the microsphere, with cells present in the biomaterial. 100 µm scale bar.
Figure 4.39: H&E stained section shows BaSO₄ loaded microspheres in a blood vessel of the (A) kidney. Enlarged view of (B) particles in adjacent blood vessels, scale bar represents 500 µm. Further enlargement (C) shows red blood cells in the microsphere, scale bar represents 50 µm.
4.3 Discussion

Suitability of the TIPS microspheres for delivery

The TIPS microspheres were formulated and optimized for non-invasive delivery. TIPS microspheres were sieved to <250 µm diameter to ensure compatibility with 23G needle delivery. Utilization of the smallest size range of <250 µm ensured that the microspheres suspension could flow through the needle and avoid occlusion. A GRANUGEL® dilution of 60% (v/v) GRANUGEL® in dH2O was optimised to ensure that the TIPS microspheres would be kept in suspension for over 4 hours. 4 hours was the estimated amount of time required between sample preparation and implantation of the product in the pre-clinical setting in the available facilities. It is likely that in a hospital setting the required suspension time might be longer, particularly if the formulation is prepared offsite and needs to undergo quality control prior to administration.

The microsphere suspension was divided into 3 equal volumes of 50 µl for intramyocardial delivery. The volume and delivery sites were chosen based on previous studies, as stated in section 4.1.1, that have shown safety and tolerability of the same administration parameters in rat models of myocardium infarction²⁹,²⁹⁰,²⁹². Intramyocardial delivery of cells has been shown to have therapeutic superiority compared to other delivery mechanisms, such as epicardial injections²⁹²,²⁹³. Gerbin et al. delivered human embryonic stem cell derived cardiomyocyte microtissue-particles (1000 hESC-CM aggregated per micro-tissue particle) by intramyocardial injection into the left ventricular wall or implanted in the epicardium of ischaemia/reperfusion injured athymic rat hearts⁷. In the study, cells delivered by intramyocardial injections showed improved maturity by cellular electrical coupling and the formation of gap junctions compared to the epicardium implants²⁹²,²⁹³. Therefore, the current study aimed to deliver 3 equal volumes of 50 µl 2% 7507 TIPS microspheres in a 60% GRANUGEL® dilution to the basal, mid, and apical region of the left ventricle.

The formulation parameters were quantified to establish the number of TIPS microspheres deliverable. The median diameter of wetted TIPS microspheres was 78 ± 12.33 µm (Chapter 3). This size range (minimum 42.4 µm, 25th percentile 68.05 µm, median 78 µm, 75th percentile 83.63 µm, maximum 104.05 µm) of TIPS microspheres was found to be suitable for delivery via 23G needle with inner
diameter of 641 µm. The number of microspheres delivered in each dose of 3 x 50 µl was quantified in Table 4.2. Except for dosage 1, the number of TIPS microspheres delivered per dose had a high variability and ranged between 352 and 2275 microspheres. The average number of TIPS microspheres delivered per treatment was 4198 ± 544. The high variability in TIPS microspheres number per dosage is likely attributed to an uneven mixing of TIPS microspheres in the GRANUGEL® dilution. The variability needed to be improved to achieve a consistent therapy, because once cells are seeded on the TIPS microspheres, the difference in delivered therapy will be amplified further. This was improved in further experiments (Chapter 7) by utilization of a syringe mixing tube which enabled mixing of dilutions while minimising the formation of air bubbles.

Impact of TIPS microspheres implantation on the in vivo model
The TIPS microspheres were delivered in the rat myocardium to evaluate persistence and biodistribution. The animals were divided into two groups, the first group received injections of 3 × 50 µl TIPS microspheres suspension into the myocardium wall, the target site, and the second group received the same formulation but into the left ventricular cavity. The second group was included to investigate the impact of microspheres entering coronary vasculature and the potential risk of systemic embolization. Since the myocardium wall of a rat is 2-3mm thick, the TIPS microspheres were delivered using ultrasound guided injections for targeted delivery.

In vivo implantation of the TIPS microspheres progressed without fatalities and the animals recovered without side effects. Assessment of cardiac function did not show significant changes post-injection and after recovery for both treatment groups. This suggested that implantation of the TIPS microspheres did not affect cardiovascular health nor had any visible side effects.

This study was the first to report the delivery of TIPS microspheres to the heart, using a minimally invasive delivery technique. The findings can inform the development of other microcarrier based products for minimally invasive delivery to the heart. Image-guided minimally invasive delivery of the microspheres is superior to open chest delivery, negating the need for an invasive surgery and the associated
risks. Additionally, the findings informed the design of further experiments in this chapter and chapter 7.

Post-injection ultrasound scans of the heart suggested implantation of the TIPS microspheres through hyperenhancement at the injection site. The hyperenhancement suggested delivery of a microspheres rather than air bubbles. Air bubbles also show on ultrasound scans as hyper enhanced speckles, but as care was taken to remove air bubbles from the TIPS microsphere formulation prior to injection, they are an unlikely cause of the speckles visible upon injection.\textsuperscript{294}

However, the microspheres-driven hyperenhancement on the US scan was an indicative suggestion and a more sensitive method for tracking the microspheres was sought. Traditionally, histological techniques have been used to evaluate biomaterial implantation. However, these techniques do not allow for real-time analysis, are semi-quantitative, invasive and do not provide functional assessment. Hence, an imaging technique was sought to enable non-destructive, longitudinal, and quantitative analysis of TIPS microsphere delivery.

**Trackable TIPS microspheres show a promising application**

To progress the *in vivo* experiments, trackable TIPS microspheres were produced. Initial attempts were made to manufacture fluorescently labelled microspheres using ICG. ICG is a tricarbocyanine dye that has been clinically approved for multiple applications, including for cardiac applications.\textsuperscript{295–297} The dye is cheap, safe and has both absorptions and fluorescence spectra in the near infrared region, enabling visualisation in deep tissue cavities.\textsuperscript{295,297,298} Attempts were made to encapsulate ICG in the TIPS microspheres during the fabrication process. Despite the microspheres appearing visibly green, which was suggestive of ICG loading, they produced a weak fluorescent signal that was not suitable for *in vivo* detection. The reason for this is unclear but might relate to degradation of the dye by the solvents used during the TIPS manufacturing process. Coating the TIPS microspheres with ICG produced a measurable fluorescent signal, with the strongest signal produced when the ICG was dissolved in 10% ethanol and added to coating media. However, the signal was below the detection limit for *in vivo* application, and this was likely due to ICG degradation by ethanol, as shown in Figure 4.11.
Therefore, an alternative approach was investigated that involved encapsulating BaSO\textsubscript{4} contrast agent in the TIPS microspheres for CT tracking. BaSO\textsubscript{4} is a radiopaque contrast media clinically utilised to examine the gastrointestinal track. This agent is insoluble and typically administered as 2\% (w/v) suspension in water\textsuperscript{299}. This formulation was embedded in the TIPS microspheres during synthesis to produce 2\% BaSO\textsubscript{4} 2\% 7507 TIPS microspheres and contrast was measured against the control TIPS microspheres without BaSO\textsubscript{4}. The loaded TIPS microspheres produced mean contrast of -526 Hounsfield Units (HU) which was higher than the control -1000 HU, but below the contrast level of soft tissue. The loading of BaSO\textsubscript{4} was increased to obtain a contrast measurement above the level of soft tissue (100 HU) and bone (1000 HU)\textsuperscript{300}. 4, 6 and 20\% BaSO\textsubscript{4} loaded TIPS microspheres were produce and the 20\% BaSO\textsubscript{4} loaded TIPS microspheres formulation produced an adequate contrast of 2024 HU.

The suitability of 20\% BaSO\textsubscript{4} TIPS microspheres formulation for \textit{in vivo} tracking was further analysed by measuring the contrast of the suspension in the delivery formulation and after a simulated dynamic degradation. In the GRANUGEL\textsuperscript{®} dilution, the 20\% BaSO\textsubscript{4} TIPS produce a visible contrast, and this was retained after degradation in moving cell media for 7 days, producing a contrast signal of 1904 HU. The simulation was done on a constantly rotating rotor to simulate \textit{in vivo} movement. Although dynamic simulation is superior to a static simulation, it did not reflect the complex components and sheer forces the TIPS microspheres will endure in the \textit{in vivo} model. Despite the limitations of the model, the data suggested that the 20\% BaSO\textsubscript{4} TIPS microspheres could be tracked up to 7 days \textit{in vivo} and this is the first time the TIPS microspheres show promise for live tracking, for this application and more.

\textit{In vivo} data showed that 20\% BaSO\textsubscript{4} loaded TIPS microspheres could be tracked for up to 6 days after minimally invasive intra-myocardial injections. Whole body and \textit{ex vivo} organ CT scans revealed that most microspheres were retained in the heart. A minority of microspheres were detected in off-target sites, including lung, kidney and liver. The lungs were the most likely implantation off-site as it was expected that microspheres entering the circulation would eventually become embedded in smaller pulmonary vessels. In off-site locations the microspheres were embedded in blood vessels, further suggesting that off-site delivery occurs from escape of the
microspheres out of the myocardium and into the left ventricular cavity. The presence of microspheres in the brain was not expected. Ex vivo brain analysis was performed on only two specimens because the study did not anticipate for this off-target site and therefore the brains were not excised. Hence, it would be crucial to repeat these experiments with analysis of more brains.

There is no existing literature reporting the delivery CT trackable microcarriers intramyocardially, but the study by Kedziorek et al. is the most comparable. Kedziorek et al. used alginate-poly-L-lysine alginate microcapsules embedded with 5%, 10%, 30%, or 70% (w/v) BaSO$_4$. Microcapsules produced a contrast signal relative to the quantity of BaSO$_4$ embedded, however the signal cannot be compared to the 20% BaSO$_4$ TIPS microspheres as the study by Kedziorek et al. used signal intensity measures and contrast to noise ratios. This study used Hounsfield units. Kedziorek et al. delivered 10% BaSO$_4$ loaded microcapsules into the left superficial femoral artery of a rabbit model of peripheral arterial disease. 10% BaSO$_4$ loaded microcapsules did not produce a contrast signal above the contrast of bone, unlike the 20% BaSO$_4$ TIPS microspheres. A high contrast biomaterial was required in our study as whole-body CT scans were acquired to discern distribution and off-target delivery. Kedziorek et al. did not report a foreign body response to the implantation of 10% BaSO$_4$ loaded microcapsules and an inflammatory infiltrate score of 1-3 (mild- severe infiltrate). This aligns with the histology results.

Overall, these experiments aimed to produce a trackable microsphere formulation to track microsphere distribution after ultrasound-guided intramyocardial injections. It is important to re-iterate that the 20% BaSO$_4$ TIPS microspheres are not comparable to the chemical profile of the 2% 7507 TIPS microspheres. However, these findings suggest that BaSO$_4$-loaded microspheres can be used as a novel tool for optimising delivery techniques and tracking persistence and distribution of implanted products.

For future pre-clinical studies, the fabrication of the BaSO$_4$ TIPS microspheres could be modified to reduce toxicity. The detrimental health effects of barium compounds have been shown to be dependent on the solubility of the compounds in water and bodily fluids. Ba$^{2+}$ ion and the soluble compounds of barium are generally highly toxic in vivo. However, the insoluble barium compounds, such as BaSO$_4$ are generally non-toxic. To reduce the toxicity of the BaSO$_4$ TIPS microspheres,
alginate encapsulation or hydrogel delivery could be explored, in order to reduce the rate of \( \text{BaSO}_4 \) particle dissolution off the TIPS microspheres\textsuperscript{216,302}. The modifications would be dependent on the desired application and target site.
4.4 Summary
The chapter aimed to assess the feasibility of delivering TIPS microspheres in the left ventricular myocardium of rodent models using ultrasound-guided injections, to provide a minimally invasive delivery method. Additionally, a trackable microsphere formulation was sought to visualise the grafted material and be used to verify injection success, retention, and distribution of the microspheres.

- A delivery formulation for the injection of TIPS microspheres was determined
- TIPS microspheres were delivered in the top, mid and basal regions of the myocardium of a rat model using ultra-sound guidance, a novel method to administer the microspheres in a minimally invasive manner. Delivery did not affect cardiac function nor induce any visible side effects.
- A trackable TIPS microsphere formulation was sought to improve the monitoring of the therapeutic in vivo
- ICG loading on TIPS microspheres produced fluorescent microspheres, but the emitted fluorescence was too low to use in vivo.
- BaSO$_4$ loading in TIPS microspheres successfully produced CT trackable microspheres in vitro and in vivo. This is the first ever reported example of trackable TIPS microspheres.
- In vivo administration of BaSO$_4$ loaded microspheres enabled tracking of the therapeutic up to 6 days. Tracking was precise, where single microspheres could be tracked in real time.
- Majority of the BaSO$_4$ loaded microspheres were retained in the heart, but a low level of microspheres was visualised in off-target sites.

Figure 4.40 provides a graphical summary of Chapter 4. TIPS microspheres were successfully formulated for non-invasive delivery and preliminary studies suggested that acellular microspheres can be delivered into the myocardium without negative effects on cardiac function. Hence, the microsphere formulation was taken forward to cellularisation and characterisation (Chapter 5 and 6), for in vivo delivery of a cellularised product (Chapter 7).
Figure 4.40: Graphical summary of Chapter 4.
Chapter 5: *In vitro* biological characterisation of iPSC and cellularised TIPS microspheres

5.1 Introduction

After establishing a process for attaching iPSC to TIPS microspheres it was necessary to demonstrate that iPSC growth characteristics and pluripotency were not affected by interaction with the microspheres.

The sensitive nature of iPSC pluripotency is impacted by changes in the cellular microenvironment, metabolism and signaling pathway. This sensitivity creates challenges in the development of cell therapies that require a desired cellular phenotype. Examples of the limitations of iPSC culture on iPSC phenotype and heterogeneity have been investigated and reviewed by Yamanaka *et al.*

Retention of iPSC pluripotent phenotype after attachment to the TIPS microspheres was sought, to enable differentiation of the attached iPSC into iPSC-CM. Hence, characterisation of iPSC phenotype is essential during the development of an iPSC-derived therapeutic.

The International Stem Cell Banking Initiative has provided recommended guidelines for the registration of clinical-grade iPSC, including: (1) pluripotency tests; (2) differentiation tests *in vitro* and *in vivo*; (3) karyotype analysis to demonstrate genetic stability; (4) determination of cell identity; (5) gene expression profiling by stem cell array; and (6) microbiological tests. These guidelines can also be used to guide the design and characterisation of pre-clinical iPSC-derived therapies as described below.

5.1.1.1 Pluripotency markers

iPSC can be functionally characterised by their ability to differentiate into other cells. Assessment of several molecular markers can identify the pluripotent status of iPSC. Human pluripotent cells express the cell surface proteins TRA-1-60, SSEA4, alkaline phosphatase and the transcription factors OCT4, SOX2 and NANOG (Figure 5.1). These markers can be verified using different analytical methods.
Figure 5.1: Schematic representation of iPSC cardiac differentiation with corresponding molecular markers. Created using BioRender.

The International Stem Cell Banking Initiative recommends immunophenotyping using flow cytometry for a minimum of two markers from the standard pluripotent stem cell panel (Figure 5.1)\textsuperscript{305}. The two markers should be combination of one intracellular (i.e. OCT4, SOX2 or NANOG) and one extracellular (i.e. SSEA4 or TRA-1-60) marker\textsuperscript{305}. In preclinical studies, these markers have also been identified using immunocytochemistry, RT-PCR and Western blots\textsuperscript{306,307}.

Alkaline phosphatase staining is another routinely used assay, as high levels of alkaline phosphatase is a marker of pluripotency\textsuperscript{306}.

5.1.2 Characterisation of cell viability
Anoikis and its significance to cell therapy were discussed in section 1.5. Previous studies, have suggested that anoikis is a major pathway that can limit the efficacy of cellular transplantation\textsuperscript{154}. Generally, when cell-based therapies are processed for \textit{in vivo} delivery, the cell component is detached from its growth surface and suspended for hours before transplantation. Whilst in suspension, essential adhesion-dependent signals mediated by integrin receptors are lost, triggering anoikis and the cells undergo apoptosis before reaching the recipient\textsuperscript{157,158}.

Hence, the TIPS microspheres aim to protect the cells from anoikis by providing an implantable substrate for tissue engineering. This chapter characterises the biomaterial-derived protection from anoikis.
5.1.2.1 Markers of anoikis

Anoikis consists of numerous cellular responses to the lack of adhesion that trigger a range of apoptotic pathways\textsuperscript{308}. Therefore, differentiating between anoikis and anoikis-independent apoptosis is difficult. The only inherent difference between the two, is how apoptosis is triggered. Anoikis is triggered by cellular detachment and disrupted integrin signalling\textsuperscript{153}.

There are several assays available for the characterisation of anoikis. These assays rely on seeding the cells of interest on ECM/Hydrogel coated or non-coated plates to mimic anoikis conditions and anchored-controls\textsuperscript{309–311}. Resulting cell viability is determined by fluorometric and colorimetric readings of live and dead cell markers (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT], calcein and ethidium bromide)\textsuperscript{309–311}. These assays are targeted towards investigating anoikis resistance in cancer cells and do not provide detailed insight into anoikis-specific mechanisms\textsuperscript{311}. An anoikis specific assay does not exist and a method to assess the mechanistic characterisation of biomaterial-derived protection from anoikis is needed.
5.1.3 Aims and Objectives

Aim 1: Demonstrate that iPSC attached to TIPS microspheres retain their required biological behaviour.

Objective 1: Quantification of cell surface markers characteristic of undifferentiated iPSC phenotype will be compared in cells pre- and post-attachment to TIPS microspheres to ensure their phenotype has not deviated following cell attachment.

Aim 2: Demonstrate that iPSC attachment to the TIPS microspheres protects from anoikis.

Objective 1: Quantification of cellular viability of iPSC and iPSC-derived products pre- and post-attachment to TIPS microspheres to provide a more sensitive and informative assay.

Objective 2: Characterisation of anoikis susceptibility of iPSC and iPSC-CM pre- and post-attachment to TIPS microspheres will increase the likelihood that TIPS microspheres protect attached cells from anoikis.
5.2 Results

5.2.1 Characterising iPSC cell proliferation

To characterise the effect of iPSC attachment to TIPS microsphere on iPSC biological activity, iPSC proliferation on the microspheres was quantified. iPSC were seeded on control tissue culture (TC) plastic conditions, consisting of 5 µg/ml VTN-N coated 6-well TC plate, or 20 mg 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N. The number of microspheres seeded was normalised to the surface area of the substrate. The attachment time point of 2 hours, was selected from the results obtained in chapter 3. Light microscopy analysis showed that after 1 hour of seeding, iPSC attached to the microspheres but had a round morphology, suggesting non-complete attachment. After 2 hours of seeding the iPSC appeared attached, with cellular spreading over the surface of the TPS microspheres. Cellular proliferation was further monitored for a total of 7 days post-seeding. The proliferation of iPSC seeded on TC plastic (Figure 5.2 A) was comparable to the proliferation of iPSC seeded on TIPS microspheres (Figure 5.2 B). After 2 hours of seeding, $0.86 \times 10^5 (\pm 0.14 \times 10^5 \text{ SEM, n}=3)$ and $0.07 \times 10^5 (\pm 0.02 \times 10^5 \text{ SEM, n}=3)$ iPSC attached to TC plastic and TIPS microspheres respectively. In both conditions, cell number increased linearly up to day 2, and between day 2-5 the cell number increased exponentially. Peak cell number was reached on day 5, mean attached cell count on TC plastic was $36.3 \times 10^5 (\pm 0.10 \times 10^5 \text{ SEM, n}=3)$ and mean attached cell count on TIPS microspheres was $3.77 \times 10^5 (\pm 0.36 \times 10^5 \text{ SEM, n}=3)$. From day 5 to 7, the number of cells attached to either TC plastic or TIPS microspheres dropped. The downwards trend conformed with expected cell proliferation dynamics, where upon reaching confluence, the total cell number declines due to competition for space and metabolic resources.
Figure 5.2: Proliferation of iPSC on (A) tissue culture plastic and (B) TIPS microspheres over 7 days. 1.00×10^5 and 0.30×10^5 iPSC were seeded in the well of a 5 µg/ml VTN-N coated 6-well plate (TC plastic) and 20 mg of wetted TIPS microspheres respectively. Seeding densities were normalised to the surface area of the substrate (9.6 cm² for TC plastic and 2.9 cm² for microspheres). Cell counts were taken daily using a Chemometec NC200, at the same time. n=3±SEM.
5.2.2 Charactering iPSC pluripotency

5.2.2.1 Microscopic evaluation

To investigate whether attachment of the iPSC to the TIPS microspheres influenced iPSC phenotype, markers of pluripotency were investigated using an alkaline phosphatase assay and confocal microscopy. After 24 hours of attachment to the TIPS microspheres, iPSC retained alkaline phosphatase activity suggesting that they retained pluripotency (Figure 5.3).

![Alkaline phosphatase staining of (A) iPSC on control TC conditions and (B) iPSC attached to TIPS microspheres after 24 hours.](image)

**Figure 5.3:** Alkaline phosphatase staining of (A) iPSC on control TC conditions and (B) iPSC attached to TIPS microspheres after 24 hours. iPSC were seeded on control TC plastic conditions, consisting of 5 µg/ml VTN-N coated 6-well TC plate, or 20 mg 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N. Scale bar represents 100 µm.

After 24 hours of attachment to the TIPS microspheres, iPSC also showed integration and total coverage of the surface of the TIPS microspheres (Figure 5.4 Bii), as shown by DAPI and phalloidin staining. Cellular bridging and formation of clusters were also visible. Confocal microscopy also confirmed the retention of iPSC pluripotency markers after 24 hours of attachment to the TIPS microspheres, via the expression of SOX2 nuclear marker and TRA-1-60 cell surface marker (Figure 5.4 Bi).

After 24 hours of attachment to the TIPS microspheres, iPSC were migrated off TIPS microspheres onto VTN-N coated tissue culture plastic for 72 hours static incubation and stained positively for SOX2 and TRA-1-60 (Figure 5.4 C). The distribution of staining was similar to iPSC attached to TC control conditions (Figure 5.4 A).
Figure 5.4: Confocal images of iPSC in (A) tissue culture conditions, consisting of 5 µg/ml VTN-N coated 8-well chamber slide, (B) attached to TIPS microspheres and (C) migrated for 72 hours, off TIPS microspheres and onto tissue culture plastic. Cells are stained with (i) pluripotent markers SOX2 (green), TRA-1-60 (red) and DAPI (blue), or (ii) DAPI (blue) and Phalloidin (red). Scale bar represents 50 µm.
5.2.2.2 Evaluation by flow cytometry

To quantify pluripotent markers and to compare iPSC culture conditions, iPSC populations were analysed by flow cytometry. Flow cytometry was used to quantify the expression of intracellular pluripotent markers SOX2 and OCT4, and extracellular pluripotent markers SSEA-4 and TRA-1-60. The gating strategy involved the selection of live cells by negative expression of NIR dead cell stain (Figure 5.5) and gates were based on the respective FMO (Fluorescence Minus One) controls and negative cell population (Figure 5.6).

Figure 5.5: Gating strategy utilised to select live iPSC population. (A) side scatter vs. forward scatter area density plots to remove debris, (B) side scatter vs. forward scatter height density plots to remove doubles, and (C) selection of live cells by negative expression of dead cell stain.
Figure 5.6: Gating strategy utilised to select the positive population of iPSC expressing (A) SSEA-4, (B) TRA-1-60, (C) SOX2 and (D) OCT4. Representative plots showing gating set according to FMO controls and negative control cells.

The experimental strategy for assessing iPSC phenotype is summarised in Figure 5.7.
Figure 5.7: Illustration of iPSC pluripotent phenotype characterisation strategy. Flow cytometric assessment of (A) the VTN-N coating composition, (B) TIPS microsphere coating composition and (C) culture conditions, on iPSC phenotype. Panel A corresponds to the results presented in Figure 5.8, Panel B corresponds to the results presented in Figure 5.10, and Panel C corresponds to the results presented in Figure 5.12. TC coating refers to 5 μg/ml VTN-N in DPBS, TIPS coating refers to 5 μg/ml VTN-N, 11.7% EtOH in HBSS. TC control refers to iPSC cultured under standard TC conditions, consisting of 5 μg/ml VTN-N coated 6-well TC plate. Created with Biorender.
Given that the VTN-N coating composition for TC plastic and TIPS microspheres was different, the impact of VTN-N coating composition on iPSC phenotype was assessed (Figure 5.8). iPSC were cultured on 6 well tissue culture plates coated for 1 hour with either 5 µg/ml VTN-N in DPBS (TC coating), or 5 µg/ml VTN-N, 11.7% EtOH in HBSS (TIPS microspheres coating). Flow cytometric analysis revealed that iPSC cultured on TC coating conditions retained expression of pluripotency markers >95%. iPSC cultures on TIPS coating retained expression of SOX2, OCT4 and TRA-1-60 pluripotency markers >90%, which were comparable to the control group. However, SSEA-4 expression was significantly lower (mean 86.54 ± 3.84 SEM, n=3, \( P=0.023 \), two-way ANOVA, Dunnett’s post-hoc correction). Representative histograms from the quantified samples are show in Figure 5.9.

**Figure 5.8: Flow cytometric characterisation of VTN coating composition on iPSC pluripotency markers in tissue culture conditions.** iPSC were cultured on 6 well tissue culture plates coated for 1 hour with either 5 µg/ml VTN-N in DPBS (TC coating), or 5 µg/ml VTN-N, 11.7% EtOH in HBSS (TIPS microspheres coating).*P=0.023, two-way ANOVA, Dunnett’s post-hoc correction. n=3±SEM.
Figure 5.9: Flow cytometric characterisation of the impact of VTN coating composition on iPSC pluripotency markers. iPSC expression of (A) SOX2, (B) OCT4, (C) TRA-1-60 and (D) SSEA-4 compared to iPSC cultured on TIPS VTN coating.

Next, the impact of TIPS microspheres protein coating composition on attached iPSC pluripotent markers was compared (Figure 5.10 and Figure 5.11). iPSC were attached to VTN-N or FBS coated TIPS microspheres and control TC conditions, consisting of 5 µg/ml VTN-N coated 6-well TC plate. Flow cytometry revealed that SOX2 expression was significantly reduced in iPSC attached to FBS coated TIPS microspheres, compared to the TC control (mean 39.47 ± 15.28 SEM, n=3,
P=0.0006, two-way ANOVA, Dunnett’s post-hoc correction). SOX2 expression in iPSC attached to VTN-N coated TIPS microspheres was not different to the tissue culture control (mean 97.73 ± 0.549 SEM, n=3). OCT4 expression was retained in all groups and comparable to TC control expression (mean 98.80 ± 0.289 SEM, n=3). SSEA-4 expression was significantly reduced in iPSC attached to VTN-N microspheres (mean 51.63± 21.33 SEM, n=3, P=0.0059) compared to TC control, and retained in iPSC attached to FBS coated TIPS microspheres. TRA-1-60 was highly expressed in the control group (mean 95.47 ± 0.183 SEM, n=3), however expression was reduced in iPSC attached to VTN-N and FBS coated TIPS microspheres. iPSC expression of TRA-1-60 was significantly reduced after interaction with VTN-N coated- (mean 17.67 ± 9.696 SEM, n=3, P= 0.0001) and FBS coated- (mean 44.30 ± 4.257 SEM, n=3, P= 0.002) TIPS microspheres. These results suggest that iPSC phenotype was affected by a combination of TIPS microspheres interaction and protein coating composition.

**Figure 5.10: Flow cytometric characterisation of the impact of TIPS coating composition on iPSC pluripotency markers.** iPSC were attached to VTN-N or FBS coated TIPS microspheres and control TC conditions, consisting of 5 µg/ml VTN-N coated 6-well TC plate. Statistics to matched control. VTN-N **P=0.0059, ****P=0.0001. FBS **P=0.0020, ***P=0.0006. Two-way ANOVA, Dunnett’s post-hoc correction. n=3±SEM.
Figure 5.11: Flow cytometric characterisation of the impact of TIPS coating composition on iPSC pluripotency markers. iPSC expression of A) SOX2, (B) OCT4, (C) TRA-1-60 and (D) SSEA-4 compared to iPSC cultured on TIPS microspheres coated with FBS or VTN-N.
The changes in pluripotent marker expression were not dependent on the cellular passaging associated with the microspheres attachment protocol (Figure 5.12). For microspheres attachment, iPSC were detached from TC conditions and attached onto the microspheres. An attachment control was investigated, matching the passaging of cells, with re-attachment onto 5 µg/ml VTN-N coated 6-well TC plate. The attachment control was comparable to the TC control, consisting of 5 µg/ml VTN-N coated 6-well TC plate, as all pluripotent marker expression levels were >95%. iPSC migration off the microspheres and back onto 5 µg/ml VTN-N coated 6-well TC plate, resulted in reduced expression of extracellular markers SSEA-4 (mean 78.87 ± 6.209 SEM, n=3, P=0.0016, two-way ANOVA, Dunnett’s post-hoc correction) and TRA-1-60 (mean 85.33.87 ± 7.348 SEM, n=3, P=0.0003), compared to TC control expression. The migration control (matched for 2 passages with re-attachment onto VTN-N coated TC plastic) generally reduced pluripotent marker expression (>75 %); however, there was no significant difference compared to the TC control. Representative histograms from the quantified samples are show in Figure 5.13.
Figure 5.12: Flow cytometric comparison of iPSC phenotype in different culture conditions: tissue culture plastic, attachment to TIPS microspheres, after migration from TIPS microspheres and matched passaging controls. TC control refers to iPSC cultured on 5 µg/ml VTN-N coated 6-well TC plate. For microspheres attachment, iPSC were detached from TC conditions, and attached onto 5 µg/ml VTN-N coated TIPS microspheres. An attachment control was investigated, matching the passaging of cells, with re-attachment onto 5 µg/ml VTN-N coated 6-well TC plate. TIPS migration consisted of iPSC migration off the microspheres and back onto 5 µg/ml VTN-N coated 6-well TC plate. The migration control was matched for 2 passages with re-attachment onto VTN-N coated TC plastic. Statistics to matched TC control. TIPS attachment **P=0.0059, ***P=0.0001. TIPS migration **P=0.0016, ***P=0.0003. Two-way ANOVA, Dunnett’s post-hoc correction. n=3±SEM.
Figure 5.13: Flow cytometric characterisation of the effect of culture conditions on iPSC pluripotency markers. iPSC expression of A) SOX2, (B) OCT4, (C) TRA-1-60 and (D) SSEA-4 compared to iPSC cultured on TIPS microspheres and migrated off TIPS microspheres.
5.2.2.3 Evaluation by RT-qPCR

Flow cytometry analysis suggested that iPSC expression of extracellular pluripotent markers was affected by attachment to VTN-N coated TIPS microspheres. However, nuclear pluripotent marker expression was not affected by this interaction. Therefore, it was assessed whether interaction with the TIPS microspheres could drive preliminary iPSC differentiation into a specific trilineage progenitor. Verification was carried out by RT-qPCR to allow the evaluation of pluripotent and differentiation related gene expression from iPSC attached on TIPS microspheres. Gene expression was compared to an iPSC control, consisting of matched passage iPSC cultured on 5 µg/ml VTN-N coated 6-well TC plate.

The qPCR panel was designed based on the studies by Granata et al.\textsuperscript{312} and D'Antonio et al.\textsuperscript{313} The primers were optimised against positive trilineage differentiated cells. Briefly, trilineage differentiated cells were obtained from P8 iPSC cultured in 6 well plates at 30-40% culture confluence. The differentiation strategies are graphically summarised in Figure 5.14. Endoderm progenitor cells were differentiated using the method described by Ishikawa et al.\textsuperscript{248}. Ectoderm differentiation was performed by the method described by Tchieu et al.\textsuperscript{249}. Mesoderm differentiation was performed as described in section 2.3.3.1, and this method was also used in Chapter 6. Figure 5.14 A-C shows representative light microscopy images of the trilineage progenitor cells, 48 hours after differentiation. The progenitor cells displayed distinctive morphological differences, including cell shape and size. qPCR primer efficiency was checked by producing a standard curve of Ct value vs. cDNA concentration per primer pair. Reaction efficiency calculations can be found in the appendix (Figure 9.20 - Figure 9.24).
Figure 5.14: Trilineage differentiation strategy used to obtain (A) ectoderm, (B) endoderm and (C) mesoderm progenitor cells to verify the specificity of qPCR primers. Light microscopy images show the respective progenitor cells after 48 hours of differentiation. Scale bar represents 100 µm.
Analysis by RT-qPCR showed gene expression to be in part variable across biological replicates. Figure 5.15 A presents *POUF51B* (encoding OCT-4) and *PODXL*, as detected in the panel of pluripotent markers, expressed by iPSC on TIPS at $0.3683 \pm 0.1412$ SD (n=4) and $0.4596 \pm 0.2631$ SD (n=4), respectively. However, there was no significant difference in expression between iPSC on TC plastic and iPSC on TIPS microspheres.

Similarly, Figure 5.15 B shows variability in the expression of *IGF2* in the mesoderm panel. But overall, there was no significant difference in expression of mesoderm markers expressed between iPSC on TC plastic and iPSC on TIPS microspheres.

Expression of endoderm marker *SOX7* was significantly upregulated (P<0.0001, unpaired t-test, Holm-Sidak’s post hoc analysis) in iPSC on TIPS microspheres compared to iPSC on TC plastic at $0.00595 \pm 0.00043$ SD (n=4) and $0.00076 \pm 0.00039$ SD (n=4), respectively (Figure 5.15 C). There was no significant difference in expression of other endoderm progenitor markers, *GATA1* and *CDH1*, between iPSC on TC plastic or TIPS microspheres.

Figure 5.15 D shows RT-qPCR data of ectoderm markers. *COL1A1* was detected in both iPSC attachment groups, with expression levels at $0.01652 \pm 0.0114$ SD (n=4) by iPSC on TC plastic and $0.03340 \pm 0.00419$ SD (n=4) by iPSC on TIPS microspheres. iPSC on TC plastic appeared to express *COL1A1* at a lower level than iPSC on TIPS microspheres, however due to variability between biological replicates, this was not significant. Variability likely occurred from technical error associated with pipetting small liquid volumes. Expression of *PAX6* was significantly upregulated (P=0.0003, unpaired t-test, Holm-Sidak’s post hoc analysis) in iPSC on TIPS compared to the iPSC control, at $0.00505 \pm 0.00104$ SD (n=4) and $0.00016 \pm 0.00007$ SD (n=4) respectively.
Figure 5.15: Trilineage differentiation marker expression in iPSC attached to TIPS microspheres. Changes in (A) pluripotent, (B) mesoderm, (D) endoderm and (D) ectoderm gene expression in iPSC attached to TIPS microspheres compared to iPSC grown on tissue culture conditions. Gene expression was calculated relative to the expression of the house keeping gene GAPDH. C ****P<0.0001, D ***P=0.0003, unpaired t-test, Holm-Sidak’s post hoc analysis. n=3-4±SD. ±SD was used to assess effect size.
5.2.3 Charactering anoikis-dependent apoptosis in iPSC and iPSC-CM

To characterise the anoikis susceptibility of iPSC and iPSC-CM on TIPS microspheres, a combination of flow cytometry and Western blot analysis was employed. iPSC and iPSC-CM attached onto 5 µg/ml VTN-N coated 6-well TC plates were used as negative control and treatment with 10 µM staurosporine for 24 hours (for flow cytometry control) or 100 µM etoposide for 4 hours (for Western blot control) were used as positive controls for apoptosis. Anoikis-dependent apoptosis was induced in suspension samples by resuspension of iPSC and iPSC-CM in a 6-well low bind plate incubated for 1, 2, 4, 8 or 24 hours. The suspension samples were compared to cells attached to TIPS microspheres, which were incubated for 24 hours to assess TIPS microspheres-derived protection from anoikis. Representative light microscopy images of the samples and controls used for Western blot and flow cytometric characterisation of anoikis-dependent apoptosis can be found in the appendix (Figure 9.25- Figure 9.27)

For Western blot analysis, expression of caspase 3, caspase 9 and cleaved caspase 3 was assessed, as they are known downstream markers of apoptosis activation\textsuperscript{314,315}. Downstream markers were chosen to provide definite evidence of activation of apoptosis via anoikis. All measurements are reported as relative signal intensity to \textit{GAPDH}.

Western blots indicated activation of apoptosis in iPSC samples after 1 hour in suspension (Figure 5.16 B and D). Caspase 9 expression was reduced to negligible levels after 4 hours, but was not significantly different from the negative control, measuring 0.0234 relative frequency \((\pm0.011\ SEM, n=4)\) and 0.6666 \((\pm0.175\ SEM, n=4)\) respectively. Caspase 3 expression significantly was reduced after 4 hours suspension \((0.1570 \pm0.119\ SEM, n=4, P=0.0126,\ two\text{-}way\ ANOVA,\ Dunnett\’s\ post\text{-}hoc\ correction)\) compared to the negative control \((1.5648 \pm0.677\ SEM, n=4)\). Caspase 3 expression remained significantly reduced up to 24 hours in suspension \((P=0.0051\text{-}0.0044)\). Cleaved caspase 3 was expressed from 2 hours onwards \((0.2207 \pm0.212\ SEM, n=4)\), significantly increased after 4 hours \((1.3263 \pm0.843\ SEM, n=4)\).
SEM, n=4, P=0.0259) and the expression peaked at 8 hours in suspension (2.4080 ±1.011 SEM, n=4, P=0.0001).

Comparable expression patterns were seen in iPSC-CM samples (Figure 5.16 C and D). Caspase 9 expression trended to decrease after 4 hours (0.4725 ±0.1618 SEM, n=4) in suspension, however the decrease was not significantly different to the negative control (0.1737 ±0.1265 SEM, P=0.9955, n=4). Similarly, caspase 3 expression trended to decrease after iPSC-CM were suspended for 4 hours (0.6470 ±0.0943 SEM, n=4) however the decreased expression also did not reach significance (negative control 0.7094 ±0.0830 SEM, P=0.9999, n=4). Cleaved caspase 3 was expressed at the 4-hour time point (0.0853 ±0.0431 SEM, n=4) and peaked after 8 hours (0.1464 ±0.0272 SEM, n=4). The expression of cleaved caspase 3 throughout the experiment was not significantly higher than the negative control (0.0122 ±0.0044 SEM, P>0.814, n=4) and expression was lower than the positive control treated with 100 μM etoposide for 4 hours (0.6818 ±0.2256 SEM, n=4).

Attachment of iPSC and iPSC-CM to the TIPS microspheres for 24 hours appeared to reduce anoikis-induced susceptibility to apoptosis in suspension culture (Figure 5.16 A). iPSC attached to the TIPS microspheres retained higher level expression of caspase 3 and caspase 9, measuring at 2.6679 (±0.2673 SEM, n=4) and 1.5992 (±0.2989 SEM, n=4).

Similarly, culture of iPSC-CM on the TIPS microspheres retained significantly high expression of caspase 3 (9.7383 ±2.2944 SEM, n=4, P<0.0001) and caspase 9 (4.3042 ±0.5272 SEM, n=4, P<0.0001), compared to the negative control.

In both TIPS microspheres groups the expression of cleaved caspase 3 was not observed. Overall, these results suggest that apoptosis was not induced in iPSC and iPSC-CM cultured on TIPS microspheres.
Figure 5.16: Characterisation of anoikis in iPSC and iPSC-derived products pre- and post-attachment to TIPS microspheres. Western blot image of (A) cells on microspheres, (B) iPSC in suspension, (C) CM in suspension and (D-E) Quantified protein expression relative to GAPDH. Hours (h), cleaved caspase 3 (cleaved C3). Positive controls were treated with 100 µM etoposide for 4 hours. Statistics to matched negative control of iPSC and iPSC-CM attached onto 5 µg/ml VTN-N coated 6-well TC plates. *P≤0.05, **P≤0.01, ****P≤0.0001. Two-way ANOVA, Dunnett’s post-hoc correction. n=4±SEM.
Next, anoikis susceptibility was characterised by flow cytometric analysis. Apoptosis/Necrosis Assay Kit (Abcam) was used to assess apoptosis with the following markers:

- Phosphatidylserine exposure as a marker of apoptosis. Binding of the phosphatidylserine on the outer plasma membrane produces red fluorescence (Ex/Em 630/660 nm)
- Loss of plasma membrane integrity is indicative to late apoptosis and necrosis. DNA Nuclear green DCS1 is a membrane impermeable dye but will label the nucleus of damaged cells. It can be observed as green fluorescence (Ex/Em 490/525 nm).
- CytoCalcein Violet 450 dye sequesters in the cytoplasm of live cells and can be detected as blue fluorescence (Ex/Em = 405/450 nm).

iPSC and iPSC-CM were either cultured in suspension or attached to TIPS microspheres for 24 hours. Cells attached onto 5 µg/ml VTN-N coated 6-well TC plates were used as negative controls and treatment with 10 µM staurosporine for 24 hours was used as a necrotic positive control (Figure 5.17). The study indicated that the viability of cells in suspension (both iPSC and iPSC-CM) was reduced, while the expression of apoptotic and necrotic markers increased, as a function of time. The longer the cells were maintained in suspension culture, the higher the susceptibility to cell death.

Attachment of iPSC to TIPS microspheres showed comparable viability to the negative control, with mean fluorescent intensity (MFI) of 125293 (±31841 SEM, n=5) and 154757 (±39552 SEM, n=5) respectively (Figure 5.17 A). Necrotic marker expression was also comparable to the negative control, at MFI of 48995 (±3535 SEM, n=5) and 35138 (±1189 SEM, n=5) respectively. MFI of the apoptotic marker was significantly higher (115257 ±34180 SEM, n=5, P= 0.0018, two-way ANOVA, Sidak’s post-hoc correction) than the negative control (21850 ±9604 SEM, n=5), suggesting activation of apoptosis. iPSC-CM attachment to the TIPS microspheres showed significant reduction in cell viability compared to the negative control, at MFI 163472 (±9755 SEM, n=6, P= 0.0018) and 200976 (±5427 SEM, n=6) respectively (Figure 5.17 B). However, apoptotic and necrotic marker expression remained comparable to the negative control.
Figure 5.17: Flow cytometry assessment of anoikis of (A) iPSC and (B) iPSC-CM pre- and post-attachment to TIPS microspheres. iPSC and iPSC-CM were cultured in suspension up to 24 hours or attached to TIPS microspheres for 24 hours. iPSC and iPSC-CM attached onto 5 µg/ml VTN-N coated 6-well TC plates were used as negative controls. Positive controls were treated with 10 µM staurosporine for 24 hours. Statistics to matched negative control. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. two-way ANOVA, Sidak’s post-hoc correction n=4-6±SEM. Low bind (LB).
5.3 Discussion

**iPSC proliferate on TIPS microspheres**

After the composition of the microspheres was selected in Chapter 3, microsphere biocompatibility with iPSC culture was assessed. Firstly, iPSC growth kinetics was assessed by comparing cell proliferation on TIPS microspheres versus TC conditions (5 µg/ml VTN-N coated 6-well TC plates). iPSC proliferation on TIPS microspheres and TC plastic was comparable, where cell number increased linearly, followed by exponential growth and a final plateau/decline. The growth kinetics were comparable to published literature, as previously discussed in section 3.3316. Despite the comparable trend, the number of cells attached to substrate, as a percentage of seeded cells, was different. 2 hours post-seeding, 86.8% of the seeded cells attached to the TC control, while only 22.8% of the seeded cells attached to the TIPS microspheres. This may have occurred from suboptimal attachment conditions to the TIPS microspheres. iPSC were attached to TIPS microspheres under static-dynamic conditions for the first 24 hours. The static-dynamic protocol has been previously established for myoblast attachment but was not optimised for iPSC attachment. Hence, there is scope to optimise the static dynamic timings, seeding-, microspheres-, and media- volumes317–320.

Bioreactors have been reported to optimise iPSC proliferation in suspension culture, however it has been reported that the sheer stress of bioreactor culture negatively affects the stemness of iPSC317–320. In contrast, Rodrigues et al., reported the spinner flask proliferation of Gibco episomal iPSC on Synthemax II coated dissolvable microcarriers254. Spinner flask proliferation had a doubling time of more than 2 days, which was longer than the observed doubling time on TIPS microspheres, but importantly culture conditions did not affect the expression of pluripotency markers, assessed by confocal microscopy254.

**Retention of pluripotent stem cell markers after interaction with TIPS microspheres**

Biocompatibility of the TIPS microspheres was further assessed by the retention of iPSC pluripotent markers. iPSC attached to TIPS microspheres after 24 hours stained positive for alkaline phosphatase. High alkaline phosphatase activity is associated with pluripotent cells and an accepted marker for pluripotent phenotype.
Maintenance of alkaline phosphatase activity have been shown to positively correlate with the self-renewal potential of human stem cell colonies\textsuperscript{321,322}. Equally, alkaline phosphatase activity gets downregulated as stem cells initiate differentiation\textsuperscript{323}. Despite this assay being widely used and accepted as a marker of stem-ness, the importance of alkaline phosphatase in iPSC physiology remains unknown\textsuperscript{321}.

To further characterise the iPSC interaction with the TIPS microspheres, confocal microscopy was used to assess the presence of cytoskeletal and pluripotent markers. DAPI and phalloidin were used to visually assess cellular attachment to the microspheres. Interaction with the TIPS microspheres was further analysed by staining for pluripotent markers SOX2 and TRA-1-60. TRA-1-60 is a surface marker detected in pluripotent cells despite not directly responsible for cellular stem-ness\textsuperscript{324,325}. Detection of TRA-1-60 marker has been utilised to detect iPSC\textsuperscript{326,327}. Positive staining for TRA-1-60 was visible on the outline of the cellularised TIPS microspheres. The distribution of TRA1-60 expression conformed with the expression patterns utilised for iPSC selection in literature, including staining of episomal iPSC on Synthemax II coated dissolvable microcarriers\textsuperscript{254,327,328}. However, SOX2 was not expressed in all of the stained iPSC. Original iPSC colonies also did not appear to express SOX2 heterogeneously and it was possible that tissue culture control colonies contained a mixed cell population. However, given that flow cytometry analysis suggested >95% SOX2 positive cell population, it was more likely that the qualitative confocal analysis underestimated SOX2 expression.

Migrated iPSC were stained for SOX2 and TRA-1-60, and compared to a control iPSC colony grown on tissue culture plastic. TRA-1-60 expression was stronger at the edge of the colonies irrespective of treatment group. This is likely to be associated with iPSC colony morphology: iPSC colonies have round morphology with distinct outlines and enclose tightly packed cells. Cells in the colony have distinct nucleoli and a high nucleus to cytoplasm volume ratio. The high-volume ratio might contribute to the higher expression pattern of TRA-1-60 in the periphery of the colony.
To complement the qualitative confocal micrographs, a flow cytometry panel was designed to quantify the expression of pluripotent markers SOX2, OCT4, TRA-1-60 and SSEA-4.

Overall, flow cytometry analysis suggests that attachment of iPSC to TIPS microspheres affects the expression of pluripotent markers. The extent of this effect appears to be dependent on the protein coating composition and interaction with the TIPS microspheres.

Attachment of iPSC to TIPS microspheres coating (5 µg/ml VTN-N, 11.7% EtOH in HBSS) on TC plastic retained the expression of pluripotent markers above 86%. SSEA-4 expression was significantly reduced in iPSC attached to TIPS microsphere coating compared to iPSC attached to control TC coating (5 µg/ml VTN-N in DPBS). The observed results could be due to the inclusion of ethanol in the coating composition, which might affect the structural properties of VTN-N. Ethanol exposure has been found to influence the secondary conformational structure of proteins and lipids in vitro. The conformational changes lead to protein denaturation. Therefore, modification of VTN-N due to ethanol exposure in the TIPS microsphere coating solution might account for the subsequent change in cell expression profile.

Nonetheless, expression of pluripotent markers in iPSC attached to TIPS microsphere coating was above the acceptance criteria of 70% positive cells set by the Global Alliance for iPSC Therapies to characterise critical quality attributes for clinical-grade iPSC. Therefore, the process for attaching iPSC to TIPS microspheres was deemed suitable for further characterisation.

Changes in pluripotent marker expression appeared additionally dependent on iPSC interaction with the TIPS microspheres. Comparison of pluripotent marker expression profiles in Figure 5.9 and Figure 5.11 suggested that iPSC attached to substrates coated with 5 µg/ml VTN-N, 11.7% EtOH in HBSS exhibited reduced SSEA-4 expression, but the reduction was more pronounced in iPSC attached to TIPS microspheres.

The data from Figure 5.11 shows that attachment of iPSC to TIPS microspheres coated with either VTN-N, and FBS reduced the expression of pluripotent markers, but the extend of the reduction was dependent on the protein coating composition.
TRA-1-60 expression was reduced to <50% positive cell expression in both FBS and VTN-N coated TIPS microspheres. Reduction in TRA-1-60 after iPSC culture on microcarrier suspension has previously been reported in the literature. Leung et al. showed that culturing IMR90 iPSC line in agitated microcarrier cultures (Matrigel coated cellulose-based DE53 microcarriers) in stirred spinner flasks, was shear sensitive and resulted in reduced expression of TRA-1-60 to 50% positive cells\textsuperscript{320}. Interaction with the VTN-N coated TIPS microspheres also reduced SSEA-4 expression to 51.63%. The reduction in pluripotent marker expression did not appear to be dependent on cellular passaging, as matched passaging controls retained pluripotent marker expression above 70% (Figure 5.12). iPSC passaging has been shown to affect iPSC phenotype and viability by DNA damage introduction of unstable genetic variants\textsuperscript{303,304,330}. Despite reduced expression of extracellular pluripotent cell markers, the expression of pluripotent nuclear markers was retained. OCT4 and SOX2 (as well as NANOG) are key elements in the pluripotency network as they have essential roles in maintaining the pluripotent state\textsuperscript{305,331}. SSEA4 is a glycolipid antigen and TRA-1-60 is a glycoprotein antigen characteristic of human pluripotent stem cells expression\textsuperscript{305,326}. Given that expression of SSEA-4 and TRA-1-60 is indicative of pluripotent cells but not the maintenance of pluripotent state\textsuperscript{332–334}, it was examined whether the TIPS microspheres-induced changes in iPSC phenotype occurred at the level of gene expression. By confirming that attachment of iPSC to TIPS microspheres affects pluripotent marker gene expression, it would be possible to deduce the impact of TIPS microspheres interaction on iPSC phenotype with more confidence.

The qPCR panel was designed to probe for early markers of trilineage differentiation, to assess the occurrence of spontaneous differentiation of iPSC attached TIPS microspheres. Spontaneous differentiation would account for the loss of pluripotent markers observed in iPSC attached to TIPS microspheres. iPSC samples attached to TC plastic and to TIPS microspheres for 24 hours were assessed, as the time point corresponded to iPSC proliferation on the surface of the TIPS microspheres (Chapter 3).

It was originally hypothesised that a clear upregulation of differentiation markers would be detectable across the whole panel, however this was not the case. Attachment of iPSC to TIPS microspheres did not affect the genetic expression of
pluripotent markers. Pluripotent markers expression was comparable to the TC plastic control, consisting of iPSC attached on TC plastic. iPSC attachment to TIPS microspheres increased the expression of endoderm gene SOX7 and ectoderm gene PAX6, compared to the TC plastic control.

SOX7 is a member of the SOX family transcription factors which regulates embryonic development and determination of cell fate. SOX7 is expressed in primitive endoderm of last blastocysts, however its function in the endoderm lineage is still unknown. The expression of SOX7 following iPSC interaction with alternative culture substrates has not been reported. Hu et al. reported upregulation in expression of SOX7, in pacemaker cells differentiated stem cells, after interaction with a silk-fibroin hydrogel. The reported results were not comparable to this study, however they confirmed that biomaterial interaction can affect SOX7 expression.

PAX6 encodes for paired box protein 6, a regulator of gene transcription and key protein in the development of neural tissue, particularly the eye. No literature has been reported on the effect of biomaterial interaction on PAX6 expression in iPSC.

It was not possible to clearly determine whether attachment of iPSC to TIPS microspheres affects iPSC phenotype. Overall, the flow cytometry data suggested that nuclear pluripotent markers were retained after iPSC attachment to TIPS microspheres, suggesting that iPSC retained their pluripotent phenotype. This was also supported by the qPCR data.

Given that the expression of extracellular pluripotent markers, SSEA-4 and TRA-1-60 is indicative of pluripotent cells but not the maintenance of pluripotent state, the observed reduction in the expression of these markers may be associated with iPSC cytoskeletal interaction with the TIPS microspheres. Glycans, including glycolipid antigens (such as SSEA4) and glycoprotein antigens (such as TRA-1-60), mediate cell adhesion and glycan expression patterns have been found to be cell specific and adhesion specific (depending on the what the cells adhere to). The role of glycans in cell adhesion and function have been reviewed elsewhere by Sigal et al. and Hakomori, however the specific role of glycosylation, including the role of SSEA4 and TRA-1-60, in defining stem cell function and adhesion still remains unclear.
It should be noted that protein detection by flow cytometry represents the translation of genetic changes after 24 hours whilst the gene expression provides a snapshot at the time of harvesting the cells. Literature suggests that small molecule-induced generation of iPSC-derived trilineage progenitors requires 48 hours\textsuperscript{248,249}. It is possible that the selected time point, of iPSC attachment to TIPS microspheres for 24 hours, was not sufficiently long enough to detect the transition into a differentiation lineage.

It would be informative to continue the assessment of iPSC phenotype after TIPS microspheres interaction and perhaps a human cell lineage identification profiler PCR array could help identify the mechanism of a TIPS microspheres-induced differentiation process.

**TIPS microspheres protect from suspension-induced necrosis**

Western blot analysis demonstrated that cells attached to TIPS microspheres retain high expression of caspase 3 and caspase 9 at 35 and 47 kDa respectively. Conforming with this, the expression of cleaved caspase 3, an indicator of cell death, could not be detected. The results were verified against positive controls, treated with 100 µM etoposide for 4 hours which is known to activate apoptosis via G1 cell cycle arrest\textsuperscript{339}, and induced anoikis consisting of cells incubated in suspension culture for up to 24 hours. Specifically, Western blot results demonstrated that uncleaved caspase 3 and uncleaved caspase 9 levels decreased dramatically while activated forms of caspase-3 increased, when iPSC and iPSC-CM were treated with etoposide (positive control) and seeded in suspension up to 24 hours (anoikis). The flow cytometry results conform with the Western blot results, suggesting that cellular attachment to TIPS microspheres was protective from anoikis induced apoptosis and necrosis. The protective effect likely occurred by provision of an attachment substrate to maintain anti-apoptotic integrin signalling. It is possible that the study missed earlier time points of cell death and stress, as cellular interaction with TIPS microspheres was only measured after 24 hours. However, the anoikis control groups suggested that cellular measures of reduced viability and increased cell death were detectable at 24 hours.

The protective effect of TIPS microspheres assisted cell therapy against anoikis was consistent with previous publications, see section 1.5 and 1.6, however
characterisation was indirectly measured by caspase expression and cell viability. For Western blot analysis, I probed for caspase 3, caspase 9 and cleaved caspase 3, which are known downstream markers of apoptosis activation\textsuperscript{314,315}. Downstream markers were chosen to provide evidence of definite activation of apoptosis via anoikis. The data supports the hypothesis of the project, where cellular attachment by TIPS microspheres could enhance the delivery of viable cells \textit{in vivo} by protection against anoikis. Increasing the percentage of live cells post-delivery is critical to the success of the cell therapies. Previous studies have correlated symptomatic relief with higher cell viability after transplantation\textsuperscript{18,110,340,341}. Li \textit{et al.} reviewed the relationship between the short-term cell retention rate and long term improvement in cardiac function, measured by left ventricular ejection fraction (Figure 5.18)\textsuperscript{110}. Comparative studies are limited by inconsistencies arising from differences in animal models, cell type and dosage, timing of delivery and models of cardiac injury\textsuperscript{110}. Nonetheless within the limitations, data from representative cardiac cell therapy studies indicated a positive correlation between cell retention rate and improvement in left-ventricular ejection fraction\textsuperscript{110}.

![Figure 5.18: Relationship between the short-term cell retention rate and long-term improvement in cardiac function.](image)

**Figure 5.18: Relationship between the short-term cell retention rate and long-term improvement in cardiac function.** Systemic, comparative study of cell therapy retention in the heart of pre-clinical animal models, delivered by retrograde coronary venous-, intracoronary- and intramyocardial injections. Retention rates measured within 24 hours post-injection. Cardiac function was measured by left ventricular ejection fraction. Data reproduced from Li \textit{et al.}\textsuperscript{110}
Protection against suspension-induced apoptosis would be particularly important in a clinical setting, where the time lag between the preparation of the cell therapy suspension and the delivery to the patient might be hours, if not days, if prepared off-site. The longer suspension time might result in increased anoikis-induced apoptosis.

This is the first study reporting a mechanistic characterisation of biomaterial-derived protection from anoikis. The assessment of this protective mechanism can provide a framework for further studies but should also continue into assessment of markers unique to anoikis signalling. Specifically, disrupted integrin signaling is unique to anoikis-dependent apoptosis and this can be used as a marker for anoikis.

Integrin receptors are heterodimers composed of non-covalently linked α and β subunits. The integrin family includes 18 α subunits and 8 β subunits, which can assemble in 24 different heterodimers. Depending on the heterodimer composition, the receptors have different extracellular ligand properties and different downstream signaling cascades. These are summarised in Table 5.1. The specificity for ECM types is determined by the expression of specific integrin subunits. This means that the expression pattern of integrin receptors is tissue specific.

There is a lack of knowledge regarding the contribution of integrin receptors on pluripotent cell-ECM interaction, including anoikis. Despite this lack of knowledge, it has been shown that iPSC and ESC have similar integrin expression profiles. iPSC express high levels of α5, α6, αv, β1 and β5 integrin subunits, however, the integrin expression profiles are not identical between iPSC lines. Hence, different iPSC lines are likely to behave differently to ECM protein sources, such as the VTN-N coating on TIPS microspheres. This also suggests that the characterisation of anoikis will need to consider (1) the cell type and (2) the respective integrin expression profile. Therefore, sensitive characterisation methods will be required to determine the markers of anoikis and guide further improvements in therapeutic design.

In conclusion, this chapter assessed the compatibility of TIPS microspheres with iPSC culture, by assessment of iPSC growth kinetics, pluripotent markers, and cell viability after microspheres attachment. Key tools were also developed for the
analysis of iPSC pluripotency, trilineage differentiation and anoikis. The developed tools were taken forward in the next chapter, where iPSC were differentiated into iPSC-CM in order to fulfill the aim of this project: to deliver iPSC-CM on TIPS microspheres.
<table>
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<td>RhoA/ROCK</td>
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<td>iC3b and fibrinogen</td>
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Table 5.1: Integrin heterodimers and their respective extracellular ligands and downstream signalling cascades. Adapted from Santoro et al. 343
5.4 Summary
The chapter assessed the biocompatibility of the formulated TIPS microspheres with iPSC phenotype, as well as the biomaterial-derived protection from anoikis. The results suggested that:

- iPSC proliferated on TIPS microspheres and the proliferation in comparable to the growth kinetics of iPSC cultured on TC conditions.

- iPSC attachment to VTN coated TIPS microspheres retains high alkaline phosphatase activity and expression of pluripotency markers SOX2 and OCT4.

- Expression of extracellular pluripotent markers SSEA-4 and TRA-1-60 was reduced below 50% positive cells after interaction with VTN coated TIPS microspheres. This effect was dependent on protein coating composition and TIPS microspheres interaction.

- iPSC pluripotent gene markers are not affected by attachment to TIPS microspheres; however, a bigger profiler array could be considered.

- Key tools were developed for the analysis of iPSC trilineage differentiation and analysis of anoikis.

- Attachment of iPSC and iPSC-CM to TIPS microspheres in suspension culture is indicated a protective phenotype against apoptosis and necrosis, suggesting protection from anoikis.

Figure 5.19 provides a graphical summary of Chapter 5. The chapter focused on iPSC biology and viability. Having shown that TIPS microspheres allow attachment of iPSC and are protective against anoikis, Chapter 6 explored cardiac differentiation protocols and assessed differentiation of iPSC into iPSC-CM on TIPS microspheres.
Figure 5.19: Graphical summary of Chapter 5.
Chapter 6: Characterisation of iPSC-CM differentiation

6.1 Introduction

After characterising the phenotype of iPSC attached to the TIPS microspheres, this chapter sought to assess cardiac differentiation strategies for the generation of TIPS microspheres cellularised with iPSC-CM.

Recent cardiac differentiation techniques have introduced defined small molecules-based approaches that guide cells through precise developmental stages and into CM specification (Figure 6.1A). Methodological improvements mainly concentrated on mimicking the embryonic developmental signals that determine mesoderm induction (activin, Nodal, BMP, Wnt and FGF) and subsequent cardiac specification (by inhibition of Wnt, BMP and TGFβ signaling)\(^94\). These small molecules-based methods have been able to generate greater yields of iPSC-CM\(^{346}\).

Lian \textit{et al.} introduced a monolayer-based directed differentiation strategy by temporal modulation of regulators of canonical Wnt signaling (Figure 6.1B)\(^{347}\). Temporal application of glycogen synthase kinase 3 (GSK3) inhibitor, Y27632, combined with a chemical Wnt inhibitor, IWP2. This differentiation strategy produced high yields of beating CM at a high purity rate (80-98\%)\(^{347}\).

Burridge \textit{et al.} further optimised this strategy by removing the chemically undefined B27 supplement\(^94\). Burridge \textit{et al.} developed the chemically defined medium (CDM3), consisting of RPMI 1640 basal medium, l-ascorbic acid 2-phosphate and rice-derived recombinant albumin\(^94\). Along with small molecule-based induction of differentiation (CHIR99021 and Wnt-C59), the protocol produced high yields of contractile CM, with up to 85\% purity and it was effective with 11 iPSC lines tested (Figure 6.1C)\(^94\).

Lian \textit{et al.} optimised their differentiation protocol following the modifications reported by Burridge \textit{et al.}\(^94,348\). The protocol was simplified by reduction of GSK3 inhibitor and Wnt inhibitor concentrations, and removing the presence of albumin in the differentiation media (Figure 6.1D)\(^{348}\). The chemically defined, albumin free conditions produced high yields of iPSC-CM with purity >90\%\(^{348}\).
Figure 6.1: Advances in small molecule-based differentiation strategies for the production of iPSC-CM. (A) Schematic representation of the cardiac differentiation stages matched to the differentiation strategies by: (B) Lian et al.\textsuperscript{347}, (C) Burridge et al.\textsuperscript{94} and (D) Lian et al.\textsuperscript{348} Created with BioRender.

The differentiation protocols of Burridge et al.\textsuperscript{94} and Lian et al.\textsuperscript{348} are the most commonly used cardiac differentiation protocols and similarly rely on the control of Wnt signaling. The protocols have methodological differences, including cell density at initiation of differentiation, exposure time to the small-molecules and the utilisation of separate differentiation and maintenance media\textsuperscript{94,348}. Despite these differences, the protocols have led to the development of proprietary cardiac differentiation media and improved cardiac differentiation efficiency, including yield and purity.
6.1.1 Purification of iPSC-CM

CM enrichment methods have been developed to purify a greater yield of CM from differentiated cell populations. The most commonly used methods can be separated in two categories: cell-surface marker separation and metabolic-based purification.

Cardiac markers cardiac troponin T (cTNT), SIRPA and VCAM can be tagged with the appropriate antibodies and separated using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting. Metabolic purification employs the unique metabolic differences in glucose and lactate metabolism between CM and non-CM (Figure 6.2).

Figure 6.2: Biochemical differences in glucose and lactate metabolism between CM and non-CM. Adenosine triphosphate (ATP), tricarboxylic acid (TCA). Created with BioRender.
Glucose is the main source of energy for mammalian cells, however CM can use different energy sources, such as lactate and fatty acids, for their metabolism. Lactate enrichment cultivates iPSC derivatives in a glucose depleted media containing lactate, enabling the sole survival of CM (Figure 6.2B). The technique has many advantages, such as: simplicity of the procedure, ease of application, no need for cell sorting and lower cost.

6.1.2 Markers and characterisation of iPSC-CM
Despite significant advances in defining the factors critical for cardiomyogenic differentiation, the resulting cultures remain a heterogeneous mixture of cell types and sub-types. Therefore, it is essential to assess cell identity. iPSC-CM can be visually distinguished by their beating function. Molecular markers can also be used to characterise their cardiac phenotype. Early CM express Nkx2.5 and myosin heavy chain. Late CM can be distinguished by the expression of cardiac troponin (cTNT), α-actin, myosin light chain and more (Figure 6.1A).

Characterisation of iPSC-CM is most commonly done through immunophenotyping of cTNT via flow cytometry. Other cardiac markers can also be investigated and characterised with different modalities, including immunocytochemistry, RT-PCR and Western blots.

The beating function as a marker of CM maturity, can be characterised using optical imaging of calcium transients and electrophysiological techniques, such as patch clamp.

Calcium transient analysis relies on the premise that calcium signalling controls the excitation-contraction coupling the heart. Orchestrated cycling of calcium from the cytoplasm, sarcoplasmic reticulum and the sarcomere controls excitation-contraction coupling and myocardial contraction. Upon excitation-contraction coupling, depolarization along the sarcolemma and T-tubules induces opening of L-type Ca$^{2+}$ channels triggering an influx of extracellular Ca$^{2+}$. The influx of Ca$^{2+}$ triggers the release of Ca$^{2+}$ from the sarcoplasmic reticulum via ryanodine receptors. Ca$^{2+}$ binding to troponin initiates myofilament sliding and myocardial contraction. The efficiency of the calcium handling system in vivo is mediated by spatial organization of the calcium handling components, where L-type Ca$^{2+}$ channels are located in close proximity to ryanodine receptors on the sarcoplasmic reticulum.
In comparison, calcium handling in iPSC-CM is generally less efficient and suggestive of an immature calcium handling system\textsuperscript{19}. The lack of T-tubules has been found to delay Ca\textsuperscript{2+} - induced Ca\textsuperscript{2+} release mechanism because of the spatial uncoupling of L-type Ca\textsuperscript{2+} channels and ryanodine receptors\textsuperscript{341,353}. During iPSC-CM maturation in vitro, the calcium stores in the sarcoplasmic reticulum increase after the onset of spontaneous contractions\textsuperscript{341,353,354}. Although the calcium content increases, iPSC-CM typically have slower calcium dynamics, characterised with increased time to peak ad slower Ca\textsuperscript{2+} decay\textsuperscript{341,354}. Structured tissue culture substrates that improve cellular alignment have shown to improve iPSC-CM calcium cycling\textsuperscript{341,355,356}. In this project, calcium transient analysis was used to characterise the impact of TIPS microspheres culture on iPSC-CM calcium handling.

To date, methods used to differentiate, purify and characterise iPSC-CM have mainly consisted of maintaining cells in 2D culture. Where 3D culture has been adopted, such as in suspended embryoid bodies (EB), mixed differentiation efficiencies have been achieved. Branco \textit{et al.} and Yan \textit{et al.} found that 3D spheroid cultures favoured differentiation and maturation of iPSC-CM, compared to 2D culture controls\textsuperscript{357,358}. Enhanced differentiation was attributed to enhanced cell-cell interactions and recreation of spatial gradients of different signals\textsuperscript{357,358}. In contrast, Zhang \textit{et al.} and Kerscher \textit{et al.} concluded that there were no major differences in the kinetics of cardiac differentiation and gene expression of cardiac markers between 2D and 3D formats\textsuperscript{359,360}. Overall, despite the potential of 3D EB to be scaled up volumetrically, these systems have been problematic because they require extensive cell handling for the dissociation and re-aggregation of the appropriate size\textsuperscript{361}. This caused difficulties in maintaining reproducibility, cell viability, and differentiation efficiency\textsuperscript{358,361}.

Microcarriers have also been used, to a limited extent, for 3D culture and differentiation of iPSC-CM. Lecina \textit{et al.} and Ting \textit{et al.} reported high yields and high purity of iPSC-CM generated on Cytodex 1 and 3 microcarriers\textsuperscript{361–363}. However enzymatic detachment of iPSC-CM and filtration of the microcarriers, greatly reduced the cell survival\textsuperscript{362,363}. To date, an integrated biodegradable microcarrier platform for culture, differentiation and \textit{in vivo} administration of iPSC-CM does not exist.
This chapter investigates the impact of attaching and culturing iPSC on TIPS microspheres and whether this influences cell differentiation towards a cardiomyocyte lineage.

6.1.3 Aims and Objectives

Aim 1: Justify the selection of cardiac differentiation protocol and parameters

   Objective 1: Selection of a cardiac differentiation protocol will maximise the yield of differentiated iPSC-CM under chemically defined conditions.

   Objective 2: Optimisation of the differentiation parameters from the selected differentiation protocol, including cell density at the beginning of differentiation, will enable the highest CM differentiation from the studied iPSC line.

Aim 2: Demonstrate that iPSC-CM attached to TIPS microspheres retain the target phenotype

   Objective 1: Enrichment of differentiated iPSC-CM population via lactate select will ensure that a purified and known cell type is attached to the TIPS microspheres.

   Objective 2: Quantification of phenotypic features characteristic of iPSC-CM following cell attachment to TIPS microspheres and culture under differentiation conditions will ensure the target cell phenotype is not impeded by cell attachment to TIPS microspheres.
6.2 Results

6.2.1 Selecting a cardiomyocyte differentiation protocol

6.2.1.1 Differentiation using a commercially available cardiomyocyte differentiation kit

The differentiation efficiency of iPSC into cardiomyocytes varies between different iPSC lines. A critical variable for the generation of robust cardiomyocyte culture is the relative confluence at the onset of differentiation. A confluence range finding study was performed to establish the optimal iPSC confluence for the generation of visible iPSC-CM beating bundles in 2D tissue culture. iPSC were seeded on VTN-N coated tissue culture plates and expanded to reach 30, 50, 70 and 90% colony confluence at day 4 (Figure 6.3 A). Differentiation at day 4 post-seeding enabled iPSC to recover from passaging. Differentiation was performed using a commercially available kit (Thermo Fisher) adapted from the protocol developed by Burridge et al.94. The changes of differentiation media are matched to cardiac differentiation stages, Media A generates mesoderm cells, Media B generates cardiac progenitor cells and cardiac maintenance media transitions the development of early cardiomyocytes. The differentiating colonies were imaged at day 0 pre-differentiation, day 2 after exposure to Media A, day 4 after exposure to Media B, and day 8 when beating colonies were visible (Figure 6.3 B). The visualisation of beating cells was a marker of cardiomyocyte phenotype.

Colonies at 30% and 50% confluence pre-differentiation produced beating colonies from day 6 and day 8 of differentiation respectively. Colonies at 70% confluence pre-differentiation produced beating bundles from day 10 and colonies at 90% confluence pre-differentiation did not produce any beating bundles.

Hence, the data suggested that seeding episomal iPSC colonies at 30% colony confluence pre-differentiation, enabled generation of iPSC-CM beating bundles at an earlier time point.
Figure 6.3: Impact of colony confluence on iPSC differentiation into iPSC-CM. (A) Images of episomal iPSC 4 days after seeding, at (i) 30%, (ii) 50%, (iii) 70% and (iv) 90% colony confluence. Confluence was visually determined. (B) The colonies were differentiated into iPSC-CM for 10 days. Optimal confluence was about 30-50%, where cells formed beating bundles from day 6-8 of differentiation. Scale bar represents 100 μm.
6.2.1.2 Differentiation of migrated iPSC off TIPS microspheres

Pluripotent phenotype can ultimately be investigated by the ability of iPSC to differentiate. iPSC were migrated off TIPS microspheres onto VTN-N coated tissue culture plastic after 24 hours static incubation. Migrated iPSC were expanded until they formed colonies and reached 30% confluence (Figure 6.4 A) and further differentiated into iPSC-CM (Figure 6.4 B and C). The differentiated cells started beating from day 8 of differentiation (Figure 6.4 D) and retained their beating phenotype of immature cardiomyocytes for up to 40 days (Figure 6.4 E and F).

The ability of migrated iPSC to form beating cardiac bundles conforms with the results from Chapter 5, suggesting that the iPSC pluripotent phenotype is retained after interaction with the TIPS microspheres.

6.2.1.3 Attachment of pre-differentiated iPSC-CM to TIPS microspheres

Up to now, the project investigated the direct attachment of iPSC to TIPS microspheres for in situ differentiation. An alternative approach to delivering iPSC-CM in vivo using the TIPS microspheres, is differentiation in vitro and attachment to the TIPS microspheres prior to in vivo delivery.

The mixed population of iPSC-CM was seeded on VTN-N coated TIPS microspheres, under static dynamic conditions. After 24 hours, $3.54 \times 10^4 \pm 2.82 \times 10^4$ SEM, n=4) of the seeded cells attached (Figure 6.5 A) and the number of cells attached did not significantly increase after 48 hours ($4.49 \times 10^4 \pm 3.77 \times 10^4$ SEM, n=3) (Figure 6.5 B and C). The lack of expansion was expected and indicative of terminally differentiated CM, which cannot replicate. The number of cells attached at 24 hours and 48 hours, represent 17.7% ($\pm 14.1$ SEM, n=4) and 22.5% ($\pm 1.9$ SEM, n=3), respectively, of the seeded cells (Figure 6.5 C).
Figure 6.4: iPSC migration off TIPS microspheres, and cardiac differentiation. (A) iPSC migrated off TIPS microspheres onto VTN-N coated tissue culture plastic. Migrated iPSC were differentiated and imaged at (B) day 2 and (C) day 4 of cardiac differentiation. Beating bundles were visible at (D) day 8, and up to (E) day 40 of differentiation. Scale bar represents 100 µm.
Figure 6.5: iPSC-CM do not expand on TIPS microspheres. $2 \times 10^5$ iPSC-CM were seeded on 20 mg of <250 µm 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour). Light microscopy images show iPSC-CM attached to TIPS microspheres after (A) 24 and (B) 48 hours post-seeding. (C) Quantification of cell attachment suggests that the fraction of cells that attaches the microspheres, does not expand. Percentages represent attached cell count as a percentage of the seeded cell count. $P=0.34$, two-tailed non-parametric t-test with Mann-Whitney test. n=3-4±SEM.
6.2.1.4 Refining the cardiac differentiation strategy

Differentiation using the commercially available kit suggested that episomal iPSC could be differentiated into iPSC-CM. Despite this, little was known of the pathways and macro-molecules required for in vitro cardiac differentiation because of the proprietary nature of the media. Therefore, I investigated the use of the chemically defined cardiac differentiation methodology published by Burridge et al., to enable improved control and understanding of the constituents required for cardiomyocyte differentiation.364

The protocol is illustrated in Figure 6.6 A and uses a basal media (CDM3) containing RPMI 1640, L-ascorbic acid 2-phosphate and rice-derived recombinant human albumin. Differentiation is induced by small molecules, GSK3 inhibitor CHIR99021 and Wnt signalling inhibitor Wnt-C59.

The major optimisable factors for this protocol were seeding density and range of CHIR99021 efficacy. iPSC were seeded on VTN-N coated tissue culture plates and expanded to reach 30, 50, 70 and 90% colony confluence at day 4. After 4 days of pluripotent growth, the cells were treated with 5, 6, or 7 µM of CHIR99021. All groups underwent cardiac differentiation until day 10, after which the efficiency of cardiac differentiation was assessed by flow cytometry analysis of cTNT positive cells, a cardiomyocyte structural marker (Figure 6.6 B and C). Light microscopy images of differentiating iPSC-CM are included in the appendix. Optimisation of cTNT staining was performed by antibody titration and selection of the highest stain index, results included in the appendix (Figure 9.29).

Figure 6.6 B shows that treatment of iPSC colonies with 7 µM of CHIR99021 yielded a better differentiation of cTNT+ cells, compared to both 5 and 6 µM. Specifically, treatment of 30% and 50% colonies with 7 µM of CHIR99021 produced a 13.1% and 13.3% cTNT+ cell population respectively. However the yield of cardiomyocytes was lower than expected, as the published protocol reported the generation of ~80–95% cTNT+ cells in more than 200 iPSC lines tested.364
Figure 6.6: Cardiac differentiation of iPSC using the Burridge et al.\textsuperscript{94} method. (A) Graphical workflow of the differentiation and purification strategy. (B) Flow cytometric quantification of cardiac troponin positive episomal iPSC differentiated with different colony confluence and CHIR concentration. (C) Representative histograms from the quantified samples. iPSC were used for negative control and unstained iPSC-CM sample was used as a unstained control. n=1-2.
Next, it was investigated whether the low differentiation yield was dependent on the iPSC line and its inherent recalcitrant potential to differentiation.

Firstly, the pluripotency was assessed by flow cytometric analysis of pluripotent markers SOX2, OCT4, TRA-1-60 and SSEA-4, as described in Chapter 5. Flow cytometric analysis confirmed iPSC pluripotency, by >85% positive expression of pluripotent markers.

iPSC underwent cardiac differentiation by the Burridge protocol, at seeding densities of 30, 50, 70 and 90% colony confluence and treatment with 5, 6, or 7 µM of CHIR99021. The groups underwent cardiac differentiation up to day 6, after which the experiment was terminated due to the extensive cell death and irreproducibility seen in all groups. Representative images can be found in the appendix (Figure 9.30 – Figure 9.32).

Simultaneously the iPSC underwent cardiac differentiation, using the commercially available kit from Thermo Fisher at seeding densities of 30, 50, 70 and 90% colony confluence. By day 8 of differentiation, all groups formed beating bundles indicative of cardiac differentiation. Representative images can be found in the appendix (Figure 9.33).

The cardiac differentiation efficiency was characterised by flow cytometry analysis. An additional marker was added to the flow cytometry panel for the analysis of cardiac cell phenotype, by combining staining for cTNT with staining for α-smooth muscle actin (αSMA), a marker of primitive cardiomyocytes. The gating strategy was based on the selection of live cells, by negative expression of NIR dead cell stain and gates were set according to unstained and negative controls (Figure 6.7). cTNT antibody titration for panel optimisation can be found in the appendix (Figure 9.28 and Figure 9.29)
Figure 6.7: Gating strategy utilised to characterise iPSC-CMs population. (A) side scatter vs. forward scatter area density plots to remove debris, side scatter vs. forward scatter height density plots to remove doublets, and selection of live cells by negative expression of dead cell stain. (B) Selection of cTNT and α-SMA positive cell population. Representative plots showing gating set according to unstained and negative control cells.
Cells derived from episomal iPSC at lower colony confluence pre-differentiation expressed higher levels of cTNT and αSMA than cells derived from higher colony confluence (Figure 6.8). Cells derived from episomal iPSC at 30% colony confluence at the onset of differentiation expressed the highest levels of cardiac cTNT and αSMA, expression at 54% and 57.8% positive cell population respectively. The higher the colony confluence, the lower the cardiac differentiation, where the cell population differentiated from episomal iPSC at 90% colony confluence was 9.43% and 11.94% positive for cTNT and αSMA respectively. Representative histograms from the quantified samples are show in Figure 6.8.

Overall, the cardiac differentiation results suggested that episomal iPSC differentiated from 30% colony confluence using the Thermo Fisher cardiac differentiation kit, yielded the highest cardiac differentiation as measured by expression of cTNT and αSMA. Hence the aforementioned seeding density specification and differentiation protocol were selected for further experiments.
Figure 6.8: Flow cytometry quantification of episomal-iPSC derived CM expression of (A) cTNT and (B) α-SMA, differentiated from different colony confluence using the commercially available Thermo Fisher cardiac differentiation kit. n=1-2.

6.2.2 Assessing cardiac differentiation on TIPS microspheres

The potential to differentiate iPSC attached the TIPS microspheres into iPSC-CM was investigated. Schematic of the differentiation assessment is illustrated in Figure 6.9. iPSC at day 0, partially differentiated iPSC-CM, at day 2 and 4, and iPSC-CM, at day 6 and 18 of differentiation, were attached to the TIPS microspheres. After attachment, the cells continued cardiac differentiation up to day 20. i.e., if partially differentiated cells were attached at day 2, differentiation was continued by addition of Media B and maintenance media, up to day 20. Representative light microscopy images can be found in the appendix (Figure 9.34 – Figure 9.38)
After 20 days of cardiac differentiation, iPSC-CM were enzymatically detached from the microspheres and assessed for cardiac phenotype by flow cytometry analysis (Figure 6.10). The expression of cardiac markers was compared to a tissue culture control, where iPSC attached to VTN-N coated tissue culture plastic at 30% colony confluence were differentiated into iPSC-CM and maintained for 20 days. The tissue culture control group expressed 61.53% (±2.37 SEM, n=4) cTNT and 59.85% (±2.59 SEM, n=4) αSMA positive cell population.

iPSC attached to TIPS microspheres at day 0 were attached for 2 hours prior to cardiac differentiation. iPSC differentiated from day 0 on TIPS microspheres expressed significantly lower levels of cTNT and αSMA, at 5.22% (±2.85 SEM, n=4) and 5.03% (±2.54 SEM, n=4), compared to the control (P= 0.0040 and P=0.0057 respectively, One-way ANOVA, Dunnett’s post hoc analysis).

iPSC-CM differentiated from day 2 primitive streak mesodermal cells on TIPS microspheres expressed 26.46% (±18.33 SEM, n=4) cTNT and 25.98% (±19.59 SEM, n=4) αSMA positive cell population. Expression levels were lower but non significantly different to the tissue culture control group.

Day 4, cardiac committed mesodermal cells on TIPS microspheres, produced a low yield of iPSC-CM, with 2.25% (±1.87 SEM, n=4) cTNT and 2.04% (±1.79 SEM, n=4).
αSMA positive cell population. The yield was significantly lower than the tissue culture control (P=0.0025 for cTNT expression, P=0.0056 for αSMA expression, One-way ANOVA, Dunnett’s post hoc analysis).

Attachment of differentiated iPSC-CM, at day 6 and 18 post differentiation, to TIPS microspheres yielded a cell population with similar expression of cardiac markers to the control group. Cells attached on day 6 of differentiation, yielded 43.37% (±13.98 SEM, n=4) cTNT and 51.06% (±17.05 SEM, n=4) αSMA positive cell population. Cells attached on day 18, yielded 52.48% (±5.14 SEM, n=4) cTNT and 55.05% (±6.60 SEM, n=4) αSMA positive cell population.

Hence, the data suggest that iPSC or partially differentiated iPSC could not be efficiently differentiated into iPSC-CM onto TIPS microspheres using the current approach. However, attachment of pre-differentiated iPSC-CM to the TIPS microspheres enabled the retention of a cardiac phenotype, which was comparable to the phenotype of cardiac cells differentiated on tissue culture plastic.
Figure 6.10: Flow cytometric characterisation of the expression of cardiac markers (A) α-SMA and (B) cTNT, in iPSC-CM attached to TIPS microspheres. 1.5 ×10⁶ cells were seeded on 20 mg of <250 µm 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 2 hours. After two hours if the cells were partially differentiated, the media was changed to continue cardiac differentiation up to day 20. The expression of the markers was (i) quantified at day 20 and (ii) Representative histograms of quantified samples plotted against tissue culture and FMO controls. Statistics to matched control CM. **P ≤ 0.01, One-way ANOVA, Dunnett’s post hoc analysis. n=4±SEM.
At day 20, the number of cells attached to the TIPS microspheres was assessed (Figure 6.11). Cells attached to TIPS microspheres on days 0, 2, 4 and 6 of differentiation produced cell counts <2×10⁵ cells on day 20. However, iPSC-CM attached on day 18, produced the highest cell attachment on microspheres, with a count of 6.25×10⁵ cells (±1.07×10⁵ SEM, P= 0.0185 – 0.0014, One-way ANOVA, Tukey’s post hoc analysis, n=3).

Given that attachment of iPSC-CM to TIPS microspheres on day 18 produced a cellularised product with the highest cell number and expression of cardiac markers, the formulation was selected for further experiments. Importantly, the project sought to deliver a formulation of pre-differentiated iPSC-CM attached to TIPS microspheres instead of a formulation differentiated on TIPS microspheres.

![Cell attachment to TIPS microspheres at day 20](image)

**Figure 6.11: Quantification of iPSC-CM attached to TIPS microspheres after cells were attached at different stages of cardiac differentiation and terminally differentiated into cardiomyocytes up to day 20.** 1.5 ×10⁶ cells were seeded on 20 mg of <250 µm 2% 7507 TIPS microspheres coated with 5 µg/ml VTN-N and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 2 hours. After two hours if the cells were partially differentiated, the media was changed to continue cardiac differentiation up to day 20. Cell count was performed using a NC200 automatic cell counter. *P= 0.0185 – 0.0014, One-way ANOVA, Tukey’s post hoc analysis. n=3±SEM.

Next, the purity of the iPSC-CM population was improved in aim to deliver a more defined cell therapy. Both metabolic and magnetic-activated cell sorting were explored.
6.2.3 Cardiac enrichment

6.2.3.1 Metabolic purification

The mixed-cardiomyocyte like population was purified by metabolic selection, where the glucose in the CDM-3 media was replaced by lactate based on the premise that only CM in the culture would be able to use the TCA cycle to produce ATP. iPSC-CM were exposed to CDM-3L between day 10-14 of differentiation (Figure 6.6 A). Light microscopy images of the purification stages were acquired and shown in Figure 6.12. From day 12 onwards, cellular apoptosis was visible and continued up to day 16, with visible cell shrinkage, detachment and membrane rounding. The cells did not recover from day 16 and large bundles of floating cells were visible, indicative of cell stress.

The purified cells were characterised for cardiac phenotype by flow cytometry (Figure 6.13), and the results suggested that metabolic purification was not successful, with only 26.67% (±5.04 SEM, n=3) cTNT and 35.40% (±8.98 SEM, n=3) αSMA positive cell population. The expression levels were significantly lower than the non-purified control (P=0.0151 for cTNT expression, P=0.0228 for αSMA expression, two-way ANOVA, Sidak’s post hoc analysis). Therefore, an alternative purification strategy was sought to improve cardiac enrichment.
Figure 6.12: Light microscopy images of lactate purification. iPSC-CM purified from day 10 of differentiation, to day 14 using glucose free, lactate rich media. iPSC-CM were recovered in glucose containing media until day 20. Scale bar represents 100 µm.
Figure 6.13: Flow cytometric characterisation of the expression of cardiac markers (A) α-SMA and (B) cTNT, in metabolically purified iPSC-CM. iPSC-CM purified from day 10 of differentiation, to day 14 using glucose free, lactate rich media. iPSC-CM were recovered in glucose containing media until day 20. The expression of the markers was (C) quantified at day 20 after purification recovery. Statistics to matched pre-injection values. P=0.0151 for cTNT expression, P=0.0228 for αSMA expression, two-way ANOVA, Sidak’s post hoc analysis. n=3±SEM.
6.2.3.2 Magnetic selection

Magnetic separation was performed using a PSC-Derived Cardiomyocyte Isolation Kit, using the two-step protocol illustrated in Chapter 2. Briefly, the first step negatively selected the CM, as non-CM as positively collected in the magnetic field. The non-CM were collected from the MACS column, as the 1st fraction. The second step positively selected CMs, flushing out the non-CM. The flushed out non-CM were collected as the 2nd fraction. Ultimately, the magnetically selected CM, and final product, were collect as the 3rd and final fraction.

Cell count revealed that the protocol enriched on average $5.25 \times 10^5$ iPSC-CM ($\pm 1.16 \times 10^5$ SEM, n=4) from $12.2 \times 10^5$ cells ($\pm 2.39 \times 10^5$ SEM, n=4) mixed cardiomyocyte-like population (Figure 6.14 A).

Flow cytometric analysis (Figure 6.14 B) revealed significantly higher purities based on αSMA (82.58% ±3.55 SEM, n=4) and cTNT (77.18% ±3.59 SEM, n=4) expression of the cells in the 3rd fraction CM population, compared to the non-purified control expression of αSMA (68.93% ±2.51 SEM, n=4, P=0.0004, two-way ANOVA, Sidak’s post hoc analysis) and cTNT (69.93% ±1.86 SEM, n=4, P=0.0003).

In contrast, the non-CM 1st and 2nd fractions expressed significantly lower populations of cTNT, 9.25% ($\pm 2.86$ SEM, n=4, P<0.0001) and 5.77% ($\pm 2.83$ SEM, n=4, P<0.0001) respectively, and αSMA, 17.56% ($\pm 6.11$ SEM, n=4, P<0.0001) and 9.05% ($\pm 4.19$ SEM, n=4, P<0.0001) respectively, compared to the non-purified control.
Figure 6.14: Characterisation of magnetically purified iPSC-CM. (B) Flow cytometric characterisation of the expression of cardiac markers (Ci) cTNT and (Cii) α-SMA. iPSC-CM were purified on day 18 of differentiation and marker expression was quantified immediately after purification. Statistics to matched control CM. *** P < 0.001, ****P<0.0001, two-way ANOVA, Sidak’s post hoc analysis. n=4±SEM.
Flow cytometry analysis indicated that the depletion of non-CMs was effective. Magnetically enriched CMs were replated as confluent monolayers on fibronectin (FBN) and VTN-N coated 35mm tissue culture dishes, cultured for 7 days. The enriched CMs showed a typical morphology and phenotype, as indicated by the expression of cTNT, and beating was visible from day 2 onwards. Representative images of beating iPSC-CM monolayer on VTN-N coated dishes can be found in the appendix (Figure 9.39). Immunofluorescence microscopy demonstrated that magnetically selected CMs displayed high expression levels of the CM-specific markers, cTNT and NKx2.5 (Figure 6.16, Figure 6.17, Figure 6.19). The non-purified iPSC-CM monolayers exhibited lower expression of cTNT and the intensity of expression of NKx2.5 was lower (Figure 6.15 and Figure 6.18).

Figure 6.15: Confocal imaging of passaged iPSC-CM monolayer on a VTN coated dish. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.
Figure 6.16: Confocal imaging of purified iPSC-CM monolayer on a VTN coated dish. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.
Figure 6.17: Confocal imaging of an iPSC-CM on a VTN coated dish. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.
Figure 6.18: Confocal imaging of passaged iPSC-CM monolayer on a FBN coated dish. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.
Figure 6.19: Confocal imaging of purified iPSC-CM monolayer on a FBN coated dish. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.

Magnetically enriched CMs were attached to VTN-N coated TIPS microspheres and spontaneous contractions were visible from day 2 onwards, seen as spontaneous movement of microspheres in suspension. Immunofluorescence microscopy demonstrated that isolated CMs attached to TIPS microspheres also displayed high expression levels of cTNT and NKx2.5 (Figure 6.20 and Figure 6.21)
**Figure 6.20:** Confocal image of purified iPSC-CM on TIPS microspheres. Visualisation of (ii) NKx2.5 in red, (iii) DAPI in blue, (iv) cTNT in green and (i) composite images. Scale bar represents 50 µm.
6.2.4 Assessment of iPSC-CM contractility

6.2.4.1 Quantification of spontaneous contraction

The spontaneous contractions generated by the enriched iPSC-CM monolayers and iPSC-CM on TIPS microspheres were quantified, to assess the physiological functionality of the generated iPSC-CM (Figure 6.22). The spontaneous beating frequency of enriched iPSC-CM on TIPS microspheres was 0.794 Hz (±0.102 SD, n=6) and similar to the frequency of non-purified iPSC-CM on TIPS microspheres 0.867 Hz (±0.106 SD, n=5, P= 0.6369, unpaired two-tailed t-test). The spontaneous beating frequency of purified and non-purified iPSC-CM on TIPS microspheres was comparable to the beating frequency of iPSC-CM on VTN-N and FBN coated plastic and glass dishes (P=non-significant, one-way ANOVA, Tukey’s post hoc analysis. n=3-6±SD). The impact of iPSC-CM purity on beating significantly affected the spontaneous beating frequency of iPSC-CM attached to VTN-N coated plastic dishes. Purified iPSC-CM on VTN-N coated plastic dishes had a significantly lower
beating frequency of 0.700 Hz (±0.067 SD, n=3, P= 0.0257, unpaired two-tailed t-test), compared to the matched non-purified iPSC-CM (0.978 Hz ±0.44 SD, n=3). iPSC-CM purity did not affect the beating frequency of iPSC-CM on FBN coated plastic dishes.

**Spontaneous cardiomyocyte contractions on different surfaces**

![Graph showing spontaneous beating frequency on different surfaces]

**Figure 6.22: Quantification of cardiomyocyte spontaneous contractions on different surfaces.** Spontaneous contractions of magnetically selected or non-purified iPSC-CM replated as confluent monolayers or on TIPS microsphere were quantified over a 30 second period using a brightfield microscope. Intrigoup statistics (purified vs. non-purified for each substrate group): *P= 0.0257, unpaired two-tailed t-test. Between group statistics: P = non-significant, one-way ANOVA, Tukey’s post hoc analysis. n=3-6±SD. ±SD was used to assess effect size in groups with different n numbers.

6.2.4.2 Optical mapping assessment of calcium transients

The function of purified iPSC-CM was further assessed by characterization of calcium handling using optical mapping. The calcium transients of spontaneous and electrical field stimulated iPSC-CM at 0.7 Hz, 1.0 Hz and 1.5 Hz were compared. FLUO-4AM fluorescence indicated the presence of calcium fluxes (data now shown). Calcium transient analysis revealed that the re-plated iPSC-CM on control monolayer conditions and on TIPS microspheres showed physiological calcium cycling (Figure 6.23). Representative optical calcium transients in Figure 6.23 also show that iPSC-CM on TIPS microspheres respond to a range of external electrical stimuli (0.7-1.5Hz), including the most physiological relevant frequency of 1.0 Hz
Figure 6.23: Representative calcium transient traces of purified iPSC-CM seeded as confluent monolayers on VTN-N coated-, FBN-coated plastic dishes and on TIPS microspheres. The spontaneous transient was compared to electrical field-stimulated iPSC-CM at 0.7, 1.0 and 1.5 Hz. Scale bar represents 200 ms.
Calcium (Ca\(^{2+}\)) flux analysis revealed that spontaneously beating iPSC-CM on VTN-N coated TIPS microspheres had significantly shorter time to peak, time to 50% Ca\(^{2+}\) decay and time to 80% Ca\(^{2+}\) decay compared to the control VTN-N coated tissue culture dish (p<0.0001, ordinary one-way ANOVA with Holm Sidak’s multiple comparison test, n=4-11±SEM) (Figure 6.24). Spontaneously beating iPSC-CM on TIPS microspheres showed comparable time to peak (83.0 ms ±5.4 SEM, n=4), time to 50% Ca\(^{2+}\) decay (297.3 ms ±11.9 SEM, n=4) and time to 80% Ca\(^{2+}\) decay (546.6 ms ±26.7 SEM, n=4) with spontaneously beating iPSC-CM on FBN coated tissue culture or glass dishes.

Electrical field stimulation of iPSC-CM on TIPS microspheres at 1.5 Hz showed comparable time to peak (82.1 ms ±3.8 SEM, n=10), time to 50% Ca\(^{2+}\) decay (215.6 ms ±6.1 SEM, n=10) and time to 80% Ca\(^{2+}\) decay (347.5 ms ±7.5 SEM, n=10) with spontaneously beating iPSC-CM on FBN coated glass dishes. 1.5 Hz stimulated iPSC-CM on VTN-N coated tissue culture plastic had significantly reduced time to peak compared to 1.5 Hz stimulated iPSC-CM on VTN-N coated tissue culture plastic dishes (131.7 ms ±6.6 SEM, n=3, p=0.0116), and FBN-N coated tissue culture plastic dishes (147.5 ms ±5.3 SEM, n=4, p<0.0001). Time to 50% Ca\(^{2+}\) decay was significantly reduced compared to 1.5 Hz stimulated iPSC-CM on VTN-N coated tissue culture plastic dishes (359.7 ms ±11.2 SEM, n=3, p<0.0001), and FBN-N coated tissue culture plastic dishes (380.8 ms ±32.8 SEM, n=4, p<0.0001). Time to 80% Ca\(^{2+}\) decay was also significantly reduced compared to 1.5 Hz stimulated iPSC-CM on VTN-N coated tissue culture plastic dishes (488.3 ms ±10.8 SEM, n=3, p<0.0001), and FBN-N coated tissue culture plastic dishes (465.0 ms ±3.6 SEM, n=4, p=0.0011).

At 1.0 Hz stimulation, iPSC-CM on TIPS microspheres had a time to peak of 79.4 ms (±3.6 SEM, n=10) which was comparable to the time to peak of 1.0 Hz stimulate iPSC-CM on FBN coated tissue culture glass dishes (111.0 ms ±13.0 SEM, n=4). Time to peak was significantly reduced compared to 1.0 Hz stimulated iPSC-CM on VTN-N coated tissue culture plastic dishes (173.7 ms ±5.6 SEM, n=3, p<0.0001), and FBN-N coated tissue culture plastic dishes (161.8 ms ±9.5 SEM, n=4, p<0.0001). Time to 50% Ca\(^{2+}\) decay in 1 Hz stimulated iPSC-CM on TIPS microspheres was 233.1 ms (±7.4 SEM, n=10) and was significantly reduced compared to iPSC-CM seeded on VTN-N and FBN coated tissue culture substrates.
(p<0.0002). Time to 80% Ca\textsuperscript{2+} decay in 1 Hz stimulated iPSC-CM on TIPS microspheres was 398.4 ms (±13.2 SEM, n=10) and was significantly reduced compared to iPSC-CM seeded on VTN-N and FBN coated tissue culture substrates (p<0.0004).

At 0.7 Hz stimulation, iPSC-CM on TIPS microspheres had significantly reduced time to peak (91.8 ms ±4.4 SEM, n=10), time to 50% Ca\textsuperscript{2+} decay (259.5 ms ±9.2 SEM, n=10) and time to 80% Ca\textsuperscript{2+} decay (440.2 ms ±12.5 SEM, n=10) compared to 0.7 Hz stimulated iPSC-CM on VTN and FBN coated tissue culture substrates (p<0.0408).
Figure 6.24: Calcium handing of purified iPSC-CM seeded as a monolayer on VTN-N and FBM coated substrates or on TIPS microspheres. Calcium imaging quantified measurements of time to (A) transient peak, (B) 50% Ca^{2+} decay and 80% Ca^{2+} decay. Statistics to matched iPSC-CM on TIPS microspheres. \*P \leq 0.05, \**P \leq 0.01, \****P \leq 0.0001. Ordinary one-way ANOVA with Holm Sidak’s multiple comparison test. n=2-11±SEM.
6.3 Discussion
This chapter aimed to assess cardiac differentiation strategies for the generation of TIPS microspheres cellularised with iPSC-CM. Cardiac purification strategies were also assessed to enrich the deliverable CM population. In addition to this, the parameters measured by flow cytometry analysis, confocal microscopy and optical mapping were used to draw a comparison between iPSC-CM populations.

Through the progression of cardiac differentiation, iPSC-CM progressively become more sensitive to passaging and detachment from their growth matrix. After detachment, late iPSC-CM are less likely to re-attach onto a substrate, including the TIPS microspheres. Therefore, it was essential to determine (1) which cardiac differentiation strategy to use and (2) the optimal differentiation stage for cellular attachment to the TIPS microspheres:

Selecting a cardiac differentiation strategy
Cardiac differentiation strategies were firstly assessed on 2D tissue culture conditions, and two differentiation protocols were compared: a commercially available kit from Thermo Fisher and the protocol by Burridge et al.94,364 The protocols were chosen because they both contained serum-free and xeno-free components, which was essential for the generation of non-immunogenic medical cell therapy products. The commercial kit was based on the Burridge protocol94, but the exact components are proprietary information. Therefore the use of the chemically defined cardiac differentiation methodology published by Burridge et al364 was investigated.

Given that the differentiation efficiency of iPSC into cardiomyocytes varies between different iPSC lines. Two additional factors were optimised in the protocols, (1) the relative confluence at the onset of differentiation and (2) CHIR concentration for the Burridge protocol364.

Overall, differentiation using the Burridge protocol yielded poor results, with high cell death and low differentiation efficiency. The following observations applied to all iPSC lines:

1. In highly confluent wells, cells died early in the differentiation process. This was expected as the cells reached maximum confluence prior to the end of differentiation.
2. In majority of wells, extensive cell death visible from day 6/8 of differentiation.

3. Only 1 well (out of 24) was beating at day 10 of differentiation, with 13.3% of live cells positive for cardiac troponin.

In contrast, iPSC lines differentiated with the commercial kit formed beating bundles by day 10 of differentiation. See appendix Figure 9.33 – Figure 9.35. A flow cytometry panel was designed to assess the yield of cardiomyocyte differentiation, by analysis of cardiac markers α-SMA and cTNT. The panel design provides a novel tool to interrogate iPSC-CM cellular phenotype, which can be used in conjunction with the iPSC 4 marker panel described in chapter The integrated nature of the panel was novel, allowing the simultaneous analysis of iPSC and CM phenotype with 6 fluorophores and could be used as a tool to guide further research into iPSC-CM differentiation. Flow cytometric analysis concurred with the light microscopy observations as detailed in section 6.2.1. Using the Burridge protocol, cardiac differentiation at day 10 was variable, as seen by difference in % cTNT positive cells between replicate plates. It is possible that the differentiated population was analysed too early in the differentiation protocol, however this is unlikely as the paper published Burridge et al. suggested that cTNT expression could be seen at day 10. The protocol paper published by Burridge et al. suggested that differentiation problems could be attributed to low passage iPSC. The authors noticed that early passage lines started dying late in the differentiation and iPSC lines above passage 25 differentiated better, with >80% cTNT positive cells. However, poor differentiation was also observed in episomal iPSC at low passage number <P10 and in higher passage numbers, >P40. It’s possible that a better cardiomyocyte yield could have been observed with further optimisation of the protocol, however given the time limitation of this PhD project, it was decided to use the commercial kit for further differentiation experiments. By utilising the commercial cardiac differentiation kit, iPSC displayed a cardiac phenotype by day 10, evidenced by the formation of beating bundles. Flow cytometry results confirmed cardiac differentiation and confirmed that episomal iPSC seeded at 30% confluence at the onset of differentiation, yielded the highest cTNT and α-SMA expressing population at day 10 post-differentiation. The cardiac differentiation parameters were optimised to inform the selection of a cardiac differentiation strategy and guide further experiments in the remainder of the project.
Determining the differentiation stage for cellular attachment to the TIPS microspheres

The project investigated the direct cardiac differentiation of iPSC on the TIPS microspheres to produce an integrated biodegradable microcarrier platform for culture, differentiation of iPSC-CM.

An alternative approach to delivering iPSC-CM in vivo using the TIPS microspheres, was differentiation of iPSC into iPSC-CM in vitro and attachment to the TIPS microspheres prior to in vivo delivery. The mixed population of iPSC-CM was seeded on TIPS microspheres coated with 5 µg/ml VTN-N. After 24 hours, only 17.7% of seeded cells attached and with great variability. Extending the attachment time to 48 hours did not significantly increase iPSC-CM attachment as only 22.5% of the cells attached. The lack of expansion was expected from a CM population, as CM do not replicate. The attachment rate was comparable to the iPSC attachment rate measured in Chapter 5, of 22.8% of seeded cells 2 hours post-seeding. It is possible that the TIPS microspheres required further optimisation of the static-dynamic conditions used to attach cells (e.g. volume of medium, ratio cells:microspheres, shaking speed and duration etc) and/or a different protein coating to promote better iPSC-CM attachment. It was assumed that VTN-N would provide a suitable protein coating for iPSC-CM attachment to the microspheres, as the iPSC used for the generation of iPSC-CM attached to VTN-N coated TC plastic and are further differentiated on the same VTN-N coating. However, in tissue culture conditions iPSC-CM attachment is typically achieved through fibronectin coating. Fibronectin is one of the major extra cellular matrix proteins that promotes cardiomyocyte attachment and therefore might be a more suitable coating protein for the microspheres that could be investigated in future work365.

This set of preliminary results suggested that attachment of pre-differentiated iPSC-CM to TIPS microspheres was possible and informed the upcoming experiments.

Direct cardiac differentiation of iPSC on the TIPS microspheres was investigated using the Thermo Fisher cardiac differentiation kit. Light microscopy images showed the differentiation of iPSC in TC control conditions from day 0, up to day 6. From day 6 the cardiac committed cells formed 3D beating structures. The critical differentiation stages and media change- time points, at day 0,2,4 and 6 of differentiation, were used to investigate whether iPSCs or partly differentiated CM
could be attached to the TIPS microspheres. After attachment, the differentiation was continued up to day 20 and the cardiac phenotype of the cells was characterised using flow cytometry.

Approximately 60% of the cells differentiated on control tissue culture plastic surfaces expressed cardiac markers cardiac troponin and smooth muscle alpha actinin. To date, the method devised does not appear to enable iPSC or partly differentiated CM attached to the microspheres to differentiate into cardiac troponin and smooth muscle alpha actinin expressing cells. However, cells pre-differentiated, to day 6 and day 18, attached to the microspheres and expressed cardiac troponin and smooth muscle alpha actinin at levels comparable to cells differentiated on TC plastic. Attachment of day 18 pre-differentiated iPSC-CM to TIPS microspheres enabled the highest cell attachment. Therefore, this formulation was taken forward as we sought the delivery of the highest cell count to maximise the likelihood of cell replacement therapy.

The iPSC-CM phenotype on TIPS microsphere was comparable to published literature. Lecina et al. investigated the expansion of hESC on microcarriers, followed by cardiomyocyte differentiation by changing hESC maintenance media to differentiation media\textsuperscript{362}. Firstly, the authors screened for cardiac differentiation on DE-53, Cytodex-1 and 3, FACT, and TOSOH-10 microcarriers\textsuperscript{362}. Microcarrier coatings were also investigated, with different extra-cellular matrices: laminin, fibronectin, vitronectin, and Matrigel\textsuperscript{362}. Laminin coating was chosen as it resulted in the most efficient hESC attachment\textsuperscript{362}. The selection strategy was similar to the strategy described in Chapter 3 of this project. Overall, the authors found that hESC on different microcarriers produced 60-90% beating aggregates, and hESCs propagated on TOSOH-10 (protamine derivatized 10 µm beads) at the concentration of 0.125 mg/ml produced 80% beating aggregates, threefold cell expansion, and 20% of CM (determined by FACS sorting for myosin heavy chain and α-SMA expression)\textsuperscript{362}. Additionally, the authors found that differentiation on microcarriers in spinner culture reduced CM yield due to the effect of shear stress\textsuperscript{362}.

Ting et al. similarly reported low hESC-CM differentiation on Cytodex I microcarriers after culture in spinner flask during expansion, with reported 22.83% cTNT expression\textsuperscript{361}. Lower shear stress in side to side rocker expansion resulted in higher
CM yields, at 47.5% cTNT expression\textsuperscript{361}. By applying intermittent agitation in the first 3 days of cardiac differentiation, followed by continuous agitation up to day 12 of differentiation, the authors reported increased CM differentiation with 65.73 ± 10.73% cTNT positive cell expression\textsuperscript{361}. Similar results were demonstrated using IMR-90 iPSC line, with expression levels comparable to the reported results in section 6.2.2\textsuperscript{361}.

The availability of comparative literature is limited, as the reported methods used hESC, and the cardiac differentiation strategies are based on the method described by Lian et al.\textsuperscript{348}. There is no literature reporting the differentiation of iPSC on microcarriers using a xeno-free cardiac differentiation strategy. Overall, the attachment of iPSC-CM and cardiac differentiation of iPSC on TIPS microsphere has never been investigated. The proposed formulation and application for TIPS microspheres are novel findings that contribute novel knowledge to the field of biomaterial-assisted cell therapy. These findings could contribute to further development of biomaterial design for cardiac cell therapy.

**Enrichment of iPSC-CM**

Cardiac purification strategies were utilised to enrich the mixed iPSC-CM population. An enriched CM population was sought to deliver a more defined cell therapy, while minimising the delivery of non-CM. Initially, lactate enrichment was investigated given the benefits of the technique described in section 6.2.3, and the potential to enrich on the TIPS microspheres directly. However, given the negative results observed in section 6.2.3 and the time limitation with the project, metabolic selection was not further optimised. Optimisation would have included adjusting the concentration of lactate, selection of D- or L-lactate, and the duration of the metabolic selection.

Instead, magnetic purification was utilised. As detailed in section 6.2.3, both negative and positive selection were utilised to purify the iPSC-CM population. Magnetic purification successfully enriched the cardiac population, and approximately 80% of the purified iPSC-CM expressed cardiac markers cardiac troponin and smooth muscle alpha actinin. This enabled for the purification of day 18 iPSC-CM prior to TIPS microsphere attachment.
The phenotype of purified iPSC-CM on TIPS microspheres conformed with the results reported by Oh et al.\textsuperscript{366}. Following from the work of Lecina et al. described above\textsuperscript{362}, Oh et al. described the production of 48.1% cTNT positive iPSC-CM differentiated on microcarriers for 10 days\textsuperscript{366}. The authors achieved high efficiency of 83.1% cTNT positive CM after purification in lactate-supplemented medium between day 10-15 in a 500 ml bioreactor scale\textsuperscript{366}.

**Characterising iPSC-CM phenotype**

In addition to flow cytometry analysis, the cardiac phenotype of differentiated iPSC-CM was assessed by confocal microscopy, spontaneous contractility, and optical mapping. Purified iPSC-CM were seeded as monolayers on VTN-N and FNB coated tissue culture dishes to generate electrically and mechanically connected iPSC-CM for optical mapping. Non-purified monolayers were also compared.

Immunofluorescence microscopy demonstrated that purified iPSC-CM displayed high expression levels of the CM-specific markers, cTNT and NKx2.5. Purified iPSC-CM attached to TIPS microspheres also displayed high expression levels of cTNT and NKx2.5. The non-purified iPSC-CM monolayers exhibited lower expression of cTNT and the intensity of expression of NKx2.5 was lower. Overall, expression of NKx2.5 positive cells was higher than cTNT expression. This was expected given that NKx2.5 is a marker of cardiac progenitor cells, but cTNT is a marker of late CM. This expression pattern suggests that the cTNT negative iPSC-CM population is likely to be composed of early CM, however this warrants for further validation of cell phenotype, either by qPCR or flow cytometry.

cTNT expression patterns were similar to the results report by Ting et al., where the hESC-CM/microcarrier aggregates formed extensive sarcomeres that exhibited cross-striations, confirming cardiac ontogeny\textsuperscript{361}.

iPSC-CM contractile function and calcium handling was characterised by quantification of spontaneous beating frequency and calcium transient analysis. iPSC-CM on TIPS microspheres exhibited a spontaneous beating frequency comparable to the control iPSC-CM monolayers seeded on VTN-N or FBN coated tissue culture substrates. The data was suggestive that iPSC-CM on TIPS microspheres were functional and possessed the appropriate phenotype.
As previously described in section 6.1.2, calcium transient analysis relies on the premise that myocardial contraction and relaxation are orchestrated by calcium cycling through excitation-contraction coupling. Mature cardiomyocyte contraction is coordinated by an efficiency calcium handling system. In comparison, iPSC-CM typically have slower calcium dynamics, characterised with increased time to peak and slower Ca\(^{2+}\) decay, suggestive of an immature calcium handling system.

Attachment of enriched iPSC-CM to the TIPS microspheres showed significantly affected Ca\(^{2+}\) cycling, with shorter time to peak, quicker time to 50% Ca\(^{2+}\) decay and quicker time to 80% Ca\(^{2+}\) decay, compared to control monolayer cultures. The faster calcium dynamics were suggestive of improved Ca\(^{2+}\) cycling. Additionally, iPSC-CM on TIPS microspheres were paced at 0.7 Hz, 1.5 Hz and at a physiologically relevant frequency of 1.0 Hz. The ability of iPSC-CM to be paced at 1 Hz suggests that in-vivo, if iPSC-CM on TIPS microspheres engraft, they could couple at a physiologically relevant frequency. Additionally, the ability of iPSC-CM on TIPS microspheres to respond to different frequencies also shows their ability to adapt to changes in heart beating frequency.

The obtained results conform with current literature. Rao et al. reported that iPSC-CM cultured on a fibronectin coated micro-grooved PDMS scaffold displayed significantly improved sarcoplasmic reticulum Ca\(^{2+}\) handling, measured by a shortened time to peak and improved sarcoplasmic reticulum Ca\(^{2+}\) release in response to caffeine. The improved calcium handling was not associated with changes in gene expression, but brought by improved cellular alignment and more organised sarcomeres.

Hence, it is possible that iPSC-CM calcium handling was influenced by culture on TIPS microspheres and this could be used to obtain a more mature calcium handling phenotype. It would be interesting to further explore the use of TIPS microspheres as a substrate to overcome the immaturity of iPSC-CM function. Further examination of iPSC-CM maturity on TIPS microspheres could focus on quantification of Ca\(^{2+}\) extrusion, spatial organisation of calcium handling apparatus and contractile cytoskeleton, expression patterns of genes encoding proteins important in calcium regulation and assessment of metabolic maturation.
6.4 Summary
Chapter 6 aimed to assess cardiac differentiation strategies for the generation of TIPS microspheres cellularised with iPSC-CM. Purification techniques were used to enrich the cardiac population and phenotype was assessed by flow cytometry, confocal microscopy, and electrophysiology. In summary:

- iPSC were differentiated into a mixed iPSC-CM population using a commercially available differentiation kit from Thermo Fisher. Seeding density was optimised at 30% colony confluence at the onset of differentiation.
- A flow cytometry panel was designed to assess cardiac phenotype by the expression of cTNT and α-SMA cardiac markers. The panel is compatible use in union with the iPSC panel described in Chapter 4.
- iPSC differentiation into cardiomyocytes following attachment to the microspheres was investigated. With the method devised, iPSC produced a low yield of cTNT and α-SMA expressing iPSC-CM when differentiated on TIPS microspheres. However, attachment of pre-differentiated iPSC-CM to TIPS microspheres was successful.
- Magnetic selection was successfully used to enrich the iPSC-CM population attached to TIPS microspheres.
- iPSC-CM on TIPS microsphere retain target phenotype, as characterised by confocal microscopy and contractile behaviour.
- Optical mapping was used for the first time to characterise iPSC-CM on a microcarrier platform. Attachment of enriched iPSC-CM to the TIPS microspheres showed improved Ca^{2+} cycling and ability to be paced at 0.7, 1.0 and 1.5 Hz.

The results in this chapter provided a final formulation for iPSC-CM cellularised TIPS microspheres suitable for in vivo investigation in Chapter 7. Figure 6.25 provides a graphical summary of Chapter 6.
Figure 6.25: Graphical summary of Chapter 6.
Chapter 7: *In vivo* implantation of cellularised TIPS microspheres in the myocardium of a healthy NSG mouse model

7.1 Introduction

Small animal models, including mice and rats, have been developed to mimic the pathomechanisms contributing to CHF. Despite some limitations, utilisation of small animal models of CHF has helped improve understanding of CHF pathogenesis and the development of novel treatment strategies. For example, coronary artery ligation in rats, a now commonly used model to mimic HF in small animals, was originally established by Pfeffer *et al.* Development of this model demonstrated that infarct size, post-MI LV dilation and LV function were correlated. Additionally, the Pfeffer group demonstrated that post-MI treatment of with ACE inhibitor captopril improved contractile function and survival in rats. Captopril treatment was subsequently tested in large clinical trials on patients with MI, which led to improved cardiac function and patient survival. The translational studies established the standardised treatment of ACE inhibition post-MI for patients worldwide.

Mice and rats are the most commonly used preclinical animal models of CHF and share a high degree of homology to the human genome. These models typically use surgical, genetic and/or pharmacological approaches to mimic the clinical features of CHF.

7.1.1 Small animal models of HFrEF

As discussed in Chapter 1, the impaired cardiac compliance and contractile dysfunction of HFpEF are associated with diastolic dysfunction. In contrast, HFrEF is typically associated with the loss of CM from myocardial damage and is associated with systolic dysfunction. HFrEF treatment would benefit from regenerative cell therapy to replace the damaged myocardium, making animal models of HFrEF the target system to test the therapeutic potential of cellularised TIPS microspheres.

A variety of stressors have been used to provoke HFrEF in small animal models, a number of these are summarised in Figure 7.1 and a more detailed description of their characteristics is summarised in the appendix (Table 10.1). Importantly, some of these models induce HFpEF, followed by the later onset of HFrEF.
For assessment of cellularised TIPS microspheres persistence in vivo, a healthy NOD scid gamma (NSG) mouse model was used. A healthy model was chosen for a preliminary assessment of cellular viability and engraftment. This precedes assessment of the regenerative potential of the microsphere formulation in a small animal model of CHF, as the therapeutic effect will likely correlate to cellular engraftment and survival.

Figure 7.1: Schematic of stressors used to induce HFrEF in rodent models. Left ventricle (LV), right ventricle (RV), ischaemia reperfusion (I/R), left anterior descending artery (LAD). Created with BioRender.

The NSG model was chosen to avoid human allograft rejection. NSG mice are an immunodeficient strain that carry two mutations on NOD/ShiLT genetic background; severe combined immune deficiency and a complete null mutation of the IL2 receptor common gamma chain. NSG mice express defective dendritic cells and
macrophages and their immune system is mainly composed of neutrophils and monocytes detectable in peripheral blood\textsuperscript{373}.

7.1.2 Pre-clinical assessment of restoration of cardiac function

The regeneration potential of a cell therapy in small animal models of CHF is typically monitored short term (1-4 weeks post-operation) and/or long term (4-12 weeks post-operation)\textsuperscript{86,87}. The cardiac regeneration potential of a cell therapy in a preclinical animal model of CHF can be assessed by:

1. Cardiac function

An improvement in cardiac function is sought and can be assessed using non-invasive clinical imaging modalities, such as ultrasound, CT, and MRI. LVEF is the most commonly reported measurement of cardiac function, other cardiac parameters also include ventricular volume and wall thickness\textsuperscript{86,100,118}. Serial cardiac function measurements should be performed to assess the developing pathogenesis of CHF and to evaluate functional improvement from therapeutic treatment.

This project assessed cardiac function using 2D guided M-mode echocardiography. Transthoracic echocardiography was performed in anesthetized animals, in combination with ultrasound-guided injections as described in chapter 4.

2. Scar morphology

Changes in scar morphology are examined to seek regeneration at the infarct size. Particularly, reduction in scar size is sought and this can be examined \textit{in vivo} using imaging modalities such as MRI. Cardiac MRI can be used to quantify regional function, quantify chamber volumes and structure, functional imaging and coronary perfusion imaging. Particularly, Late gadolinium-enhanced (LGE) MRI has the ability to measure infarct size non-invasively prior to therapeutic treatment\textsuperscript{374}. Post-treatment, acute and late LGE MRI infarct size can be compared to assess therapeutic efficacy\textsuperscript{290}. Scar morphology can also be examined post-treatment \textit{ex vivo} through histological analysis of the infarct and border zone\textsuperscript{86,100,118}. Staining using picrosirius red and fast green is commonly used to detect the infarct zone and its size\textsuperscript{86,100,118}. More detailed immunohistochemistry can be performed to distinguish the nature of the cells at the infarct area and determine re-vascularization\textsuperscript{86,118}.
(3) Graft morphology
The efficacy of the cell therapy can be assessed by measuring the graft size at the site of implantation, and in more detail, the localization of the engrafted cells and graft phenotype through immunostaining\textsuperscript{86,87,100,118}. Engraftment with native CM has been identified using immunostaining for the co-expression of cell adhesion protein pan-cadherin and gap junction protein connexin 43 in the graft and host tissue\textsuperscript{86,100,293}. Electrical coupling as a measure of engraftment has been measured using iPSC-CM transduced with fluorescent calcium indicators and electrical mapping\textsuperscript{263,292}.

(4) Cell tracking
Cell tracking is sought to confirm cellular persistence and engraftment. Previous cell therapy studies correlated symptomatic relief with higher cell viability after transplantation\textsuperscript{18,110,340,341}. Hence, the survival rate of transplanted cells has been used as a factor to determine the outcome of translation cell therapies. Efficacy could be tested by assessment of cellular retention after administration (to initiate therapeutic effect) and the length of cell survival at the administration site. Non-invasive \textit{in vivo} imaging technologies have been used to track labelled cells and assess early response to cell therapies. Cells are labelled by two general approaches: direct cell labelling and imaging of reporter genes.

Direct cell labelling has been achieved by labelling cells with various imaging probes suitable for the desired imaging modality used. Examples include the use of near infra-red florescent dyes for fluorescent imaging\textsuperscript{375}, superparamagnetic iron oxide nanoparticles (SPIONs) for MRI tracking\textsuperscript{376}, radionuclides such as 18F-fluorodeoxyglucose for nuclear imaging\textsuperscript{377}. Direct cell labelling is mostly suited for the detection of cells shortly after delivery rather than long-term tracking as the tracers dilute with cell division. Additionally, the labels are prone to leakage and retention in dead cells.

Reporter gene imaging has been achieved by introducing the desired reporter gene into the DNA of cells pre-administration. Lentivirus are the most commonly used viral vectors for transduction. After delivery of the transduced cells \textit{in vivo}, an appropriate exogenous substrate can be administered for interaction with the reporter gene products and for production of a detectable signal. Reporter genes have been
developed for MRI\textsuperscript{378}, photo acoustic\textsuperscript{379}, nuclear\textsuperscript{380} and imaging platforms\textsuperscript{381}. This chapter specifically utilised bioluminescent imaging for iPSC-CM tracking.

7.1.3 Bioluminescent tracking

BLI is an optical imaging method that relies on the light generated by the enzyme luciferase, without the need of an excitation source. BLI imaging has been applied to translation experiments, from \textit{in vitro} assessment of cellular function, such as assessment of cell viability, to \textit{in vivo} cell tracking in small animal models\textsuperscript{382,383}.

Transduction of iPSC-CM with reporter genes enables live tracking of the cell therapy to investigate the localization and viability of the cell therapy over the treatment period\textsuperscript{381}. iPSC have been transduced with firefly luciferase expressing lentivirus and treated with neomycin to stably express firefly luciferase after cardiomyogenic differentiation\textsuperscript{381,384}. Firefly luciferase is the brightest luciferase gene and therefore the most commonly used reporter gene for cell tracking\textsuperscript{385}. Light is produced by administration of D-luciferin substrate to the \textit{in vivo} system\textsuperscript{385}. Oxidation of D-luciferin by the luciferase enzyme results in light emission at 550-570nm\textsuperscript{385}. Hence, bioluminescence can be used to track Luciferase positive iPSC-CM and measure localization and cell viability in real time.

In this project, iPSCs were transduced to express luciferase tisis x5 red 2A enzyme and enhanced green fluorescent protein (eGFP). The bright colour shifted firefly luciferase based on the thermostable x5 Fluc with maximum emission wavelength of 612nm, was used to achieve a brighter signal and deeper penetration\textsuperscript{21}. Transduction was achieved using the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP (Figure 7.2). pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP plasmid has previously achieved efficient expression of luciferase tisis x5 red 2A and eGFP as described by Aswendt \textit{et al.} and Zaw Thin \textit{et al.}\textsuperscript{382,383}
Figure 7.2: pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP vector map. iPSC were transduced with a lentiviral vector using plasmid pSEW to express eGFP and firefly luciferase tisis x5 red 2A enzyme, under the control of spleen focus-forming virus (SFFV) promoter. Open reading frame (ORF). Created with SnapGene.
7.1.4 Aims and Objectives

Aim 1: Finalise the delivery formulation for the injection of cellularised TIPS microspheres.

Objective 1: Transduction of iPSC-CM with luciferin will enable the tracking *in vivo* of the cell therapy. Tracking will demonstrate that cells delivered using TIPS microspheres have higher engraftment and survival, than cells delivered in suspension.

Objective 2: Evaluate whether viable iPSC-CM on TIPS microspheres can be effectively delivered by injection *in vitro*.

Aim 2: Demonstrate that TIPS microspheres improve the engraftment and viability of iPSC-CM in a healthy preclinical rodent model.

Objective 1: Deliver purified transduced iPSC-CM on TIPS microspheres and iPSC-CM in suspension, in the top, mid and basal myocardium of a healthy NSG mouse model using ultra-sound guidance to attain localized and retained delivery.

Objective 2: Ultrasound assessment of cardiac function will determine the changes in cardiac function after treatment of iPSC-CM delivered on TIPS microspheres vs. suspension.

Objective 3: Histological analysis of explanted heart samples, 14 days post-injection, to investigate whether TIPS microsphere delivery of iPSC-CM enhanced cell engraftment.
7.2 Results
7.2.1 Transduction with the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP
7.2.1.1 iPSC transduction and characterisation

iPSC were transduced to express luciferase and eGFP for cell sorting and BLI imaging. This was achieved using the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP. Transduced iPSC were sorted by eGFP positive expression and excluded against an eGFP negative control (Figure 7.3).

Figure 7.3: Fluorescence activated cell sorting for eGFP positive iPSC. P5 episomal iPSC were transduced with lentivirus encoding firefly luciferase and eGFP for 24 hours. After 24 hours iPSC were fed with fresh complete E8 media and cultured for 4 days. After expansion, eGFP positive cells were selected using fluorescence activated cell sorting. Gating strategies and exclusion against negative control.
The effect of lentival genome integration on cell viability and differentiation capability was assessed and no adverse effect was observed. eGFP+ Luc+ sorted iPSC were expanded and characterised by phenotype analysis. Additionally, to evaluate the effective intensity of luciferase activity, an in vitro luciferase assay was performed using BLI (Figure 7.4). Light microscopy images showed the formation of morphologically typical iPSC colonies (Figure 7.4 A); compact colonies with distinct borders, containing cells with large nuclei and less cytoplasm content. The luciferase activity of transduced iPSC was tested by monitoring the luminescence of serially diluted cells, which reflects the number of viable, metabolically active cells. Luminescent activity correlated with counts of iPSC seeded at various densities from $1.5 \times 10^5$ through $8.0 \times 10^2$ (Figure 7.4 B). A time course was performed to determine peak luciferase activity after addition of luciferin. The time course in Figure 7.4 D indicated that luminescent signal was brightest at 20 minutes after the addition of luciferin, with peak signal of $1.32 \times 10^5$ Radiance ($\pm 1.47 \times 10^4$ SEM, n=6).

Pluripotency of eGFP+ Luc+ iPSC was assessed by flow cytometric analysis of pluripotent markers SOX2, TRA-1-60 and SSEA-4, as described in Chapter 5 (Figure 7.4 C). Flow cytometric analysis confirmed >85% positive expression of pluripotent markers in transduced iPSC, however expression of TRA-1-60 and SSEA-4 was significantly reduced, at 86.99% ($\pm 5.41$ SEM, n=6, $P=0.0070$, two-way ANOVA, Sidak’s post-hoc analysis) and 88.8% ($\pm 2.96$ SEM, n=4, $P=0.0334$, two-way ANOVA, Sidak’s post-hoc analysis) respectively, compared to the non-transduced iPSC population. Transduced iPSC expression of SOX2 (96.07% $\pm 2.35$ SEM, n=6) was comparable to the control (97.73% $\pm 0.35$ SEM, n=6, non-significant, two-way ANOVA, Sidak’s post-hoc analysis).
Figure 7.4: Characterisation of transduced iPSC, after cell sorting. (A) Light microscopy images of cell and colony morphology. (B) Luminescent activity correlation and (D) time course to counts of iPSC seeded at various densities from $1.5 \times 10^5$ through $8.0 \times 10^2$. (C) Flow cytometric characterisation of transduction on iPSC pluripotency. *P=0.0334, **P=0.0070, two-way ANOVA, Sidak’s post-hoc analysis. n=6±SEM.
7.2.1.2 Generation of transduced iPSC-CM

The retention of trackable luciferase activity was then tested in iPSC-CM (Figure 7.5). Luc+ eGFP+ iPSC underwent cardiac differentiation for 10 days, as detailed in Chapter 6. Differentiated iPSC-CM formed beating bundles (Figure 7.5 A, red arrows) indicative of CM phenotype, but non-CM were also visible in the differentiated population (Figure 7.5 A, blue arrows).

Luminescent activity and the associated time course of iPSC-CM correlated with counts of iPSC-CM serially diluted at densities from $1.5 \times 10^5$ through $8.0 \times 10^2$ (Figure 7.5 B and D). The time course in Figure 7.5 D indicates that luminescent signal was brightest at 24-27 minutes after the addition of luciferin, with peak signal of $2.74 \times 10^5$ Radiance ($\pm 1.10 \times 10^5$ SEM, n=4). The timing to achieve peak signal was comparable to the iPSC timing in Figure 7.4, however the signal deviation was bigger due to variable viability of iPSC-CM at seeding (data not included). Despite the variability, transduced iPSC-CM were more luminescent than transduced iPSC, with double the peak average luminescent signal.

The cardiac phenotype of eGFP+ Luc+ iPSC-CM was assessed by flow cytometric analysis of cardiac markers cTNT and α-SMA, as described in Chapter 6 (Figure 7.5 C). Flow cytometric analysis revealed that iPSC-CM differentiated from eGFP+ Luc+ iPSC exhibited significantly lower expression of cardiac markers, cTNT (39.53% ±5.54 SEM, n=4, P<0.0001, two-way ANOVA, Sidak’s post-hoc analysis) and αSMA (39.60% ±5.51 SEM, n=4, P<0.0001, compared to the non-transduced iPSC population (cTNT 62.98% ±1.86 SEM, n=4 and αSMA 68.98% ±2.51 SEM, n=4, positive population). The lowered expression of cardiac markers was addressed by magnetic selection for iPSC-CM prior to attachment to the TIPS microspheres. Overall, Figure 7.4 and Figure 7.5 established that eGFP+ Luc+ iPSC could be differentiated into eGFP+ Luc+ iPSC-CM for tracking the cell therapy in vivo.
Figure 7.5: Characterisation of CM differentiated from transduced iPSC. (A) Light microscopy images of cell morphology and formation of beating bundles (red arrow). Blue arrow indicates non-CM. (B) Luminescent activity correlation and (D) time course to counts of iPSC-CM seeded at various densities from $1.5 \times 10^5$ through $8.0 \times 10^2$. (C) Flow cytometric characterisation of transduction on cardiac differentiation markers. ***$P=0.0001$, two-way ANOVA, Sidak’s post-hoc analysis n=4±SEM.
7.2.2 Assessing the injectable delivery of iPSC-CM on TIPS microspheres *in vitro*

7.2.2.1 Evaluation of cell attachment

The delivery of viable iPSC-CM was a property instrumental to TIPS microsphere assisted cell delivery *in vivo*. To examine whether iPSC-CM on TIPS microspheres could be effectively delivered by injection to the heart, cell survival post-injection was assessed *in vitro*. Day 18 purified iPSC-CM on TIPS microspheres were resuspended in a GRANUGEL formulation and injected through a 23G needle capped syringe or uncapped control, as described in Chapter 4. The injected microspheres were immediately analysed or cultured up to 14 days. Light microscopy images in Figure 7.6 showed iPSC-CM remained attached to TIPS microspheres up to 14 days post-injection. The cellularised microspheres also retained their spontaneous beating characteristics.

![Light microscopy images](image)

**Figure 7.6:** Light microscopy images of iPSC-CM attached TIPS microspheres after syringe injection. Day 18 purified iPSC-CM on TIPS microspheres were resuspended in a GRANUGEL formulation and injected through a 23G needle capped syringe or uncapped control. Images show iPSC-CM attachment to the TIPS microspheres, 14 days after injection through (A) uncapped syringe control and (B) 23G needle. Scale bar represents 100 μm.
iPSC-CM attachment to the TIPS microspheres post-injection was confirmed by confocal microscopy (Figure 7.7- Figure 7.10). The mechanical forces experienced during syringe needle flow did not appear to detach iPSC-CM from the TIPS microspheres, as attachment of iPSC-CM to TIPS microspheres was comparable to the no-syringe control up to 7 days (Figure 7.7).

Quantification of iPSC-CM attachment on TIPS microspheres after injection (Figure 7.8) confirmed that there was no significant difference between the 23G needle group (2.69×$10^5 \pm 1.01\times 10^5$ SEM, n=5) and the control (3.74×$10^5 \pm 7.51\times 10^4$ SEM, n=5, unpaired two-tailed student’s t-test).

However, at day 14 post-injection iPSC-CM on TIPS microspheres showed signs of cellular death with visible nuclear fragments stained in blue and irregular Phalloidin staining (Figure 7.7 Bvi).
Figure 7.7: Confocal micrographs of iPSC-CM on TIPS microspheres after injection. $1 \times 10^6$ purified d18 iPSC-CM were seeded on 20 mg of microspheres and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. The media was replaced, and the cells were left to recover for another 72 hours. 4 days after cell seeding, the sample was resuspended in 500 µl of 60% GRANUGEL and injected through a 23G needle capped or uncapped syringe. The samples were fixed and stained for nuclear (DAPI blue) and cytoskeleton (phalloidin red) markers. Scale bars represent 100 µm. Microspheres are outlined in yellow.
iPSC-CM on TIPS microspheres post-injection

![Bar chart showing cell count for uncapped control and 23G needle conditions.]

**Needle passaging condition**

**Figure 7.8:** Quantification of purified iPSC-CM attached to TIPS microspheres, post-injection through a 23G needle. 1×10^6 purified d18 iPSC-CM were seeded on 20 mg of microspheres and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. The media was replaced, and the cells were left to recover for another 72 hours. 4 days after cell seeding, the sample was resuspended in 600 µl of 60% GRANUGEL and injected through either a 23G needle capped or uncapped syringe. After injection, number of cells in the sample was quantified using a Chemometec automated cell counter. P= NS, unpaired two-tailed student’s t-test. n=5±SEM

7.2.2.2 Evaluation of cell viability

The viability of injected iPSC-CM on TIPS microspheres was assessed by staining with the Apoptosis/ Necrosis Assay Kit described in Chapter 5. Figure 7.9 suggested that the majority of iPSC-CM on TIPS microspheres pre-injection stained positive for viability marker Hoescht 33342, but with a minority of cells exhibited an apoptotic and necrotic phenotype. Confocal micrographs of stained samples pre-injection showed some evidence of apoptosis (red), but majority of cells stained positive for Hoescht, indicative of viable phenotype. Necrosis marker Nuclear green DCS1 staining colocalised with Hoescht staining, suggesting there was bleeding through the blue-green channel. However, positive DCS1 staining could be distinguished by the brighter green signal, as seen in Figure 7.9 Biv and Civ.
Figure 7.9: Confocal images of purified iPSC-CM attached to TIPS microspheres, pre-injection. (A) Large cluster, (B) small cluster and a (C) single microsphere were stained for viability, apoptosis and necrosis markers. (i) Composite image acquired from overimposing (ii) CellMask™ orange plasma membrane stain, (iii) Apopxin deep red, (iv) necrosis marker Nuclear green DCS1 and (v) viability marker Hoescht. $1 \times 10^6$ purified d18 iPSC-CM were seeded on 20 mg of microspheres and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. The media was replaced, and the cells were left to recover for another 72 hours. 4 days after cell seeding, the sample was stained. Scale bars represent 100 µm.
Confocal micrographs of iPSC-CM on TIPS post-injection through a 23G needle (Figure 7.10), showed high retention of cellular viability by positive staining for Hoescht. A minority of cells exhibited an apoptotic and necrotic phenotype, by staining positive for apopxin red and necrotic marker nuclear green DCS1. Interestingly, a cluster of apopxin red positive cells was visible in Figure 7.9 Ai and Biii, but the cluster was associated with non-attached cell clumps. The expression patterns in Figure 7.10 were comparable to iPSC-CM on TIPS pre-injection in Figure 7.9, which suggested that injection of iPSC-CM cellularised TIPS microspheres could deliver viable iPSC-CM.
Figure 7.10: Confocal images of purified iPSC-CM attached to TIPS microspheres, post-injection through a 23G needle. (A) Large clusters, (B) medium clusters and a (C) single microsphere was stained for viability, apoptosis, and necrosis markers. (i) Composite image acquired from over imposing (ii) CellMask™ orange plasma membrane stain, (iii) Apopxin deep red, (iv) necrosis marker Nuclear green DCS1 and (v) viability marker Hoescht. 1×10^6 purified d18 iPSC-CM were seeded on 20 mg of microspheres and attached under static dynamic conditions (30 seconds gentle agitation at 30 RPM every hour) for 24 hours. The media was replaced, and the cells were left to recover for another 72 hours. 4 days after cell seeding, the sample was resuspended in 500 µl of 60% GRANUGEL and injected through a 23G needle capped syringe. After injection, the sample was immediately stained and fixed. Scale bars represent 100 µm.
Overall, the results in section 7.2.2 suggested that iPSC-CM on TIPS microspheres could be effectively delivered by injection, to warrant proceeding with the *in vivo* cardiac delivery experiments.

7.2.3 *In vivo* delivery of transduced iPSC-CM on TIPS microspheres

15 male 4 weeks old NSG mice were allocated into two groups to receive ultrasound-guided injections of either iPSC-CM on TIPS microspheres or iPSC-CM in suspension. Delivery of acellular microspheres by ultrasound-guided injections was validated in Chapter 4. The study design is summarised in Figure 7.11. Treatment groups received a pre-injection assessment of cardiac function via ultrasound analysis, followed by ultrasound guided intramyocardial injections (3 boluses of 15 µl) in the top, mid or apical region of the LV myocardium and a post-injection assessment scan. The first group (n=7) received iPSC-CM in suspension (6.0 x 10^5 purified transduced iPSC-CM resuspended in 500 µl 60% GRANUGEL® solution) and the second group (n=6) received iPSC-CM on TIPS microspheres (6.0 x 10^5 purified transduced iPSC-CM were attached to 20 mg of hydrophilized 2% 7507 <250 TIPS microspheres and resuspended in 500 µl 60% GRANUGEL® solution). The animals were monitored and imaged with BLI over 14. On day 14, the cardiac function was re-assessed using ultrasound analysis and the animals were sacrificed. Histology was performed on the explanted heart to look for the presence of injected TIPS microspheres.
Figure 7.11: Diagrammatic representation of the delivery protocol for cellularised TIPS microspheres into a NSG mouse model. 20 mg of 2% 7507 TIPS microspheres <250 µm were wetted in a solution of HBSS and 5 µg/ml VTN. Transduced iPSC were expanded for 3 passages and differentiated into iPSC-CM as described in section 2.3.3.1. On day 18 of differentiation, the cells were purified as described in section 2.3.3.2. 6.0 x 10^5 purified iPSC-CM were attached to 20 mg of hydrophilized 2% 7507 <250 TIPS microspheres as described in section 2.3.4.2. After attachment the cells were resuspended in 500 µl solution of 60% GRANUGEL as described in section 2.1.5. Cell suspension injections were prepared by resuspending 6.0 x 10^5 purified iPSC-CM in 500 µl solution of 60% GRANUGEL. The materials were prepared under aseptic conditions and used within 1 hour of preparation. Treatment was composed of 3 boluses of 15 µl, for the total delivery of 45 µl of either cellularised TIPS microspheres (n=6) or iPSC-CM in suspension (n=7). The cardiac function was assessed pre-injection at day 0, by ultrasound. Animals received injections in the top, mid or apical region of the LV myocardium. The persistence of the iPSC-CM localization was assessed at day 0, 1, 4 and 7 and 14 post-injection, by whole body BLI. On day 14, the cardiac function was re-assessed using ultrasound analysis and the animals were sacrificed. The major organs, including heart, lungs, kidneys, liver, and brain, were removed for further histological analysis. Created with BioRender.
7.2.3.1 Assessment of cardiac function

Administration of transduced iPSC-CM on TIPS microspheres and iPSC-CM in suspension did not adversely affect cardiac function, as shown by no significant change in measured ejection fraction (Figure 7.12). Additional measurements of heart function were also acquired (Figure 7.13) and summarised in Table 7.1, confirming that cardiac function was not adversely affected.

Figure 7.12: Assessment of cardiac function following injection of iPSC-CM. Cardiac function was measured by ejection, before-, after- and 14 days post- cardiac injection. P= NS, Two-way ANOVA, Tukey’s post-hoc correction. n=6-7±SD.

<table>
<thead>
<tr>
<th></th>
<th>Cells in suspension</th>
<th>Cells on TIPS microspheres</th>
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<tbody>
<tr>
<td></td>
<td>Pre-injection</td>
<td>Post-injection</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>48.63</td>
<td>61.23</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
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<td>32.36</td>
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<td>LV volume, at diastole (μl)</td>
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<td>43.99</td>
</tr>
<tr>
<td>LV volume, at systole (μl)</td>
<td>23.85</td>
<td>17.43</td>
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</tbody>
</table>

Table 7.1: Summary of cardiac function analysis. Values are the means reported in Figure 7.12, n=6-7. Cardiac function was measured by ultrasound analysis, before and after cellularised cardiac injections.
Figure 7.13: Changes in (A) fractional shortening, (B) LV mass, LV volume at (C) diastole and (D) systole. Additional measures of cardiac function were measured by ultrasound analysis, before-, after- and 14 days post-cardiac injection. P= NS, Two-way ANOVA, Tukey’s post-hoc correction. n=6-7±SD
7.2.3.2 BLI tracking

The viability of iPSC-CM attached to TIPS microsphere and delivered *in vivo* was assessed by comparing BLI results with iPSC-CM delivered in a suspension. Both groups showed the majority of BLI signal located within thoracic cavity at 1 hour after injection and no obvious difference in cell distribution pattern was observed throughout the study (Figure 7.14 and Figure 7.15). To note, luminescent signal emitted from the right hindlimb was due to leakage of luciferin post-injection. Ventral cavity luminescence in Figure 7.14, day 14 was due to shaving gel.

Both groups showed a decrease in signal intensity over the chest cavity at a similar rate over the 14-day time course (Figure 7.16). Signal intensities were comparable (P=NS), except for day 4 measurements, where the BLI signal of iPSC-CM in suspension (2999 p/s/cm²/sr ±320 SEM, n=7) was significantly higher than the signal of iPSC-CM on TIPS (1715 p/s/cm²/sr ±181 SEM, n=6, P=0.033, unpaired t-test with statistical significance determined using the Holm-Sidak method).
Figure 7.14: BLI images of luciferase labelled iPSC-CM in the heart at different time points after US-guided cardiac injection. BLI images at 1 hour, days 1, 4, 7 and 14 after injection showing the highest signal within the heart at day 0 and decreasing over time. Representative ROI where measurements were taken from is shown in red.
Figure 7.15: BLI images of luciferase labelled iPSC-CM delivered on TIPS microspheres in the heart, at different time points after injection. BLI images at 1 hour, days 1, 4, 7 and 14 after injection showing the highest signal within the heart at day 0 and decreasing over time. Representative ROI where measurements were taken from is shown in red.
Figure 7.16: Quantification of average BLI signal in the chest cavity over 14 days. 15 male 4 weeks old NSG mice were allocated into two groups to receive ultrasound-guided injections of either iPSC-CM on TIPS microspheres or iPSC-CM in suspension. The first group (n=7) received iPSC-CM in suspension (6.0 x 10^5 purified transduced iPSC-CM resuspended in 500 µl 60% GRANUGEL® solution) and the second group (n=6) received iPSC-CM on TIPS microspheres (6.0 x 10^5 purified transduced iPSC-CM were attached to 20 mg of hydrophilized 2% 7507 <250 TIPS microspheres and resuspended in 500 µl 60% GRANUGEL® solution). The persistence of the iPSC-CM localization was assessed at day 0, 1, 4 and 7 and 14 post-injection, by whole body BLI. *P=0.033, Unpaired t-test with statistical significance determined using the Holm-Sidak method. n=6-7 ±SEM.

7.2.3.3 Histology

Haematoxylin and eosin stained tissue sections of explanted heart tissue confirmed implantation of TIPS microspheres in the left ventricle (Figure 7.17 and Figure 7.18). 2-4 microspheres were found per explanted heart tissue and an immune response was seen around the site of microsphere implantation (Figure 7.19).
Figure 7.17: H&E stained heart sections show the implantation of cellularised TIPS microspheres in the left ventricular wall of a NSG mouse. (A) Tissue cross section shows the location of the implantation site. Yellow arrows in (B and C) the higher magnification images indicate the presence of the microsphere. 100 µm scale bar.
Figure 7.18: H&E stained heart sections show the implantation of cellularised TIPS microspheres in the left ventricular wall of a NSG mouse. (A) Tissue cross section shows the location of the implantation site. Yellow arrows in (B and C) the higher magnification images indicate the presence of the microspheres. Green arrow indicates the infiltration of cells inside the microspheres. 100 µm scale bar.
Figure 7.19: H&E stained heart sections show the inflammation caused by the needle track. (A) Tissue cross section shows the location of inflammation, near the implantation site. (B-D) The higher magnification images show the presence of immune cells at the site of inflammation. 100 μm scale bar.
7.3 Discussion
The aim of this chapter was to assess the use TIPS microspheres for delivery of iPSC-CM in vivo, in a pre-clinical rodent model.

**Transduction of iPSC for BLI tracking**
Firstly, iPSC were transduced with the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP to obtain a polyclonal iPSC population expressing luciferase tisis x5 red 2A and eGFP. eGFP expression was used to monitor successful transduction and for eGFP+ cell sorting. Luciferase expression was sought for in vivo BLI tracking. This was the first ever reported example of successfully transduced Gibco episomal iPSC for transgene expression of luciferase tisis x5 red 2A and eGFP. The cell line would also be useful to any investigations assessing iPSC and iPSC-based cell therapies, by providing cell viability and tracking information. The transduced iPSC could also be used as a tool to guide further research outside of cardiac applications. The transduced iPSC could be differentiated into additional cell types to investigate cell therapies in non-cardiac applications.

Retention of iPSC phenotype after transduction was confirmed by flow cytometry analysis. Transduction significantly reduced expression of pluripotent markers SSEA-4 and TRA-1-60 from >95% to >85% compared to the non-transduced control. However, the value was above the acceptance criteria of 70% positive cells set by the Global Alliance for iPSC Therapies to characterise critical quality attributes for clinical-grade iPSC.\textsuperscript{305} Generation of transduced iPSC meant that the cells could be used for further differentiation experiments. Light microscopy images also showed the formation of morphologically typical iPSC colonies.

Transduced iPSC were differentiated into iPSC-CM, however the efficiency of cardiac differentiation was significantly reduced compared to non-transduced iPSC. The low differentiation efficiency likely occurred as an effect of lentiviral transduction, rather than experimental variation. Experimental variation was controlled by utilisation of matched passage number, the same differentiation media and assessment of 4 separate biological replicates. Differentiated eGFP+ Luc+ iPSC-CM formed beating bundles indicative of CM phenotype, but non-CM were also visible in the differentiated population. Flow cytometry analysis confirmed that the
differentiated population exhibited lower expression of cardiac markers, cTNT and αSMA compared to the non-transduced iPSC population (39% and 60% respectively). However, the lower cardiac differentiation yield was overcome by magnetic selection for iPSC-CM.

Most importantly, luciferase activity was confirmed in transduced iPSC and derived iPSC-CM. The time course of luminescence of serially diluted cells, both transduced iPSC and iPSC-CM, determined that *in vitro* luminescence signal was brightest at 20-24 minutes after the addition of luciferin. Overall, the signal emitted by transduced iPSC-CM was brighter than the signal emitted by transduced iPSC. The luminescence data corroborated with published literature\textsuperscript{387–389}, confirming the limitations of stable transgene expression, which in this case was observed by reduced luciferase signal in iPSC.

Stable transgene expression from retroviral vectors is limited by silencing events. Silencing is caused by different mechanisms, including promoter methylation, repressive histone modifications and integration site. The integration site can lead to vector silencing and variegation of transgene expression by spreading of suppressive neighbouring heterochromatin in the integration locus. These effects are commonly observed in numerous cell types, however silencing events are more pronounced in iPSC\textsuperscript{387}. Vector silencing has been attributed to defence mechanisms against viral DNA, specific to iPSC\textsuperscript{387–389}. Overall, the described silencing events contribute to the difficulty of iPSC transduction and stable transgene expression, which were observed in this project.

Hoffmann *et al.* compared different retroviral systems and promoter configurations for transduction of iPSC and their differentiated endothelial progeny for expression of eGFP\textsuperscript{387}. The authors found that the lentivirus was the most effective vector system at transducing iPSC and they authors hypothesize that this was attributed to inherent capacity of lentiviruses to actively cross the nuclear membrane and, thus, also transduce slowly and in non-dividing cells\textsuperscript{387}. However, transgene expression levels were lower from the potent SFFV promoter, which was the same promoter used in this chapter\textsuperscript{387}. Hoffmann *et al.* attributed the reduced expression levels to an iPSC-specific reduction of SFFV promoter activity (compared with HT1080 cells), an otherwise strong viral SFFV promoter\textsuperscript{387}. The lower eGFP expression appeared to
be directly related to silencing events as insertion of UCOE (ubiquitous chromatin opening element, with known anti-silencing function) improved expression\(^3^8^7\). Additionally, transgene expression was higher after differentiation of transduced iPSC into endothelial cells\(^3^8^7\). Hence, the published results conform with the difference in luminescence observed between transduced iPSC and iPSC-CM. The signal emitted by transduced iPSC-CM was brighter than the signal emitted by transduced iPSC due to iPSC specific transgene silencing events. Despite the difference in signal, a cell product was obtained for BLI tracking in vivo.

**Injectable delivery of cellularised TIPS microspheres**

Prior to in vivo delivery, it was verified whether viable iPSC-CM on TIPS microspheres could be effectively delivered by injection in vitro. Direct injection is the preferred method of delivering cell therapy due to its minimally invasive nature\(^8^5,3^4^0,3^9^0\). Increasing the percentage of live cells post-injection is critical to the success of the cell therapies. Previous studies correlated symptomatic relief with higher cell viability after transplantation\(^1^8,1^1^0,3^4^0,3^4^1\). However, current delivery protocols using direct injection result in poor cell viability, with viabilities as low as 1–32% post-injection\(^3^9^1\). The first point of cell damage occurs through mechanical disruption of cells during the injection procedure\(^1^5^9\). During syringe needle flow, cells experience three types of mechanical forces that can lead to cell disruption: (i) a pressure drop across the cell, (ii) shearing forces due to linear shear flow, and (iii) stretching forces due to extensional flow\(^1^5^9,3^9^0\). Aguado et al. systematically compared the effects of shear stress with extensional flow (the deformation that evolves from stretching along streamlines, experienced during needle ejection) to shear stress alone, on injected HUVEC viability. The authors tested resuspension of HUVECs in Newtonian fluids (PBS and glycerol), viscoelastic fluids (non-crosslinked alginate solutions), on their in vitro model of cell injection\(^3^9^0\). The authors demonstrated that during cell injection, mechanical membrane disruption resulted in acute loss of cellular viability\(^3^9^0\). The damage was attributed to extensional flow at the entrance of the needle. The authors further demonstrated that cells (HUVEC, adipose stem cells and rat mesenchymal stem cells) are protected from the damaging mechanical effects of extensional flow by encapsulation within an alginate hydrogel\(^3^9^0\). This suggests that delivery of the cell-laden microspheres in the
GRANUGEL dilution could also protect the iPSC-CM from the damaging mechanical effects of extensional flow.

To determine the impact of cell carrier mechanics on cell viability post-injection, purified iPSC-CM on TIPS microspheres were loaded into the GRANUGEL dilution, previously described in Chapter 4, and injected through a 23G needle capped syringe or uncapped syringe control. Light microscopy images in Figure 7.6 showed iPSC-CM remained attached to TIPS microspheres up to 14 days post-injection and retained their beating phenotype. iPSC-CM attachment was confirmed by confocal microscopy and attachment of iPSC-CM to TIPS microspheres was comparable to the uncapped syringe control up to 7 days. Quantification of iPSC-CM attachment on TIPS microspheres immediately after injection suggested a trend towards a decreased count of iPSC-CM on TIPS microspheres after injection through the 23G needle. The results could be attributed to cell detachment off the microspheres; however, the trend did not reach significance compared to the control. The viability of injected iPSC-CM on TIPS microspheres was assessed by staining with the Apoptosis/ Necrosis Assay Kit described in Figure 7.9 and Figure 7.10. Overall, expression patterns of live, dead and apoptotic markers of iPSC-CM on TIPS microspheres post-injection (Figure 7.10) were comparable to iPSC-CM on TIPS pre-injection (Figure 7.9), which suggested that injection of iPSC-CM cellularised TIPS microspheres could deliver viable iPSC-CM.

The results conform with literature published by Lin et al. where injection of chondrocyte laden chitosan/PEGDA hydrogel microspheres through a 21G needle did not produce a significant decrease in cell viability\textsuperscript{392}. Using an injection rate of 5 ml h\textsuperscript{−1}, the results showed that cell viability was around 80% after injection, and there was no significant decrease in cell viability after further culture for 7 days\textsuperscript{392}. Cell viability was measured by qualitative Live/Dead assay kit and the survival rate of chondrocyte on microspheres was measured by fluorescence microscopy\textsuperscript{392}.

Overall, the \textit{in vitro} viability experiments suggested that iPSC-CM on TIPS microspheres could be effectively delivered by injection, which enabled me to proceed with the \textit{in vivo} cardiac delivery experiments.
**In vivo delivery of cellularised TIPS microspheres**

Transduced iPSC-CM laden TIPS microspheres were delivered to 4-week-old male NSG mice. The NSG model was chosen to avoid human allograft rejection. The animals were allocated into two groups to receive ultrasound-guided injections of either transduced iPSC-CM on TIPS microspheres or control iPSC-CM in suspension. Ultrasound analysis of cardiac function confirmed that both treatment groups did not adversely affect cardiac function, measured post-injection and at day 14 of post-injection. Graft-associated ventricular arrhythmias have been observed after iPSC-CM transplantation in vivo, but only in big animal models\(^{85,393}\). These arrhythmias have been shown to originate from the transplanted CM, acting as ectopic pacemakers\(^{86}\). Most importantly, graft-associated ventricular arrhythmias were not identified in smaller animal studies, nor in this study\(^{19,85}\).

Despite ultrasound analysis suggesting that microsphere implantation did not adversely affect cardiac function, it is possible that the immune response, elicited by microsphere implantation, might be physiologically detrimental. Figure 7.17 - Figure 7.19 showed a presence of an immune response at the implantation site, which was likely to consist of neutrophils and monocytes as previously reviewed by Anderson and Shive\(^{394}\) (Figure 7.20). The detrimental impact of an inflammatory response on cardiac structure could be further assessed by cardiac MRI. Cardiac MRI is the gold standard technique to quantify and monitor alterations in cardiac structure and function, providing reliable information of cardiac architecture and physiology.

![Figure 7.20: Inflammatory and healing response to the implantation of biodegradable PLGA microspheres. Reproduced from Anderson and Shive\(^{394}\).](image-url)
The cell delivery was tracked by BLI for luminescent luciferase activity. Both groups showed a decrease in signal intensity over the chest cavity at a similar rate over the 14-day time course. The comparable results suggested that TIPS microsphere attachment did not improve the viability of delivered cells. However, it is important to note that BLI imaging is not very sensitive, and the transduced cells were not very bright compared to the background signal. Therefore, sensitive changes in cell numbers could not be measured. The luminescent signal from suspension iPSC-CM was brighter than the signal from cell laden microspheres, however this more likely reflected a difference in cell count. The number of cells delivered in the treatment and control group were equalised to $6.0 \times 10^5$ purified day 18 iPSC-CM per 500 µl, with or without TIPS microspheres. However as detailed in Chapter 5 and 6, not all the iPSC-CM attach to the TIPS microspheres and therefore the microspheres group likely contained a lower number of deliverable cells.

Histology was performed to determine whether any of the microspheres were retained in the wall of the ventricle. H&E stained tissue sections of explanted hearts confirmed the presence of TIPS microspheres in the left ventricle. Only a few microspheres were found in each of the explanted hearts examined, which may indicate that either very few microspheres were actually delivered into the heart or that they were not retained following injection. Obstruction of microspheres at the neck of the needle might have reduced the number of microspheres delivered by injection. This was not observed in previous in vivo studies in Chapter 4 using acellular microspheres, however cellularisation of the microspheres might have made the microspheres stickier and more prone to aggregation. Additionally, the mechanical action of the heart also increases the risk of wash out and leakage of cells and biomaterials with every cardiac cycle, potentially resulting in cell low retention and leakage in the thoracic cavity. Unfortunately, I was unable to further assess for iPSC-CM implantation due to time-limitations. Further histological validation is required to show cellular engraftment, particularly for identification of transduced iPSC-CM by immunohistochemistry analysis for eGFP and human troponin antibody.

Overall, I was unable to conclude whether TIPS microspheres delivery could improve iPSC-CM engraftment and survival. Given the limitations of the experiment, the
The project would greatly benefit from repeating the experiment with the following changes:

1. **Improved BLI tracking**
   BLI imaging is not very sensitive, and the transduced cells were not very bright. Single cell sorting for the brightest eGFP+ transduced iPSC population might produce a more luminescent monoclonal cell population for more accurate tracking. Utilisation of a monoclonal population would likely reduce contamination with non-transduced cells that might hamper the total luminescent signal.

2. **Appropriately matched cell number**
   Control cell number was matched to the number of cells seeded on the microspheres. However, the number of cells attached to the microspheres prior to in vivo delivery was not quantified and given that attachment efficiency was <100%, the number of delivered cells was likely lower. The number of iPSC-CM delivered in the suspension control should be matched to the number of cells attached to the microspheres prior to in vivo delivery.

3. **Sensitive detection method for microsphere localisation**
   Histological analysis detected a few microspheres per heart and a more sensitive method for tracing the microspheres is required. In Chapter 4, 3×50µl of BaSO₄ loaded microspheres were delivered to a rat heart. CT tracking enabled the visualisation of numerous BaSO₄ loaded microspheres in the heart. However, the number of microspheres detected by H&E staining was lower than expected, suggesting that histology might underestimate the number of delivered microspheres. Utilisation of trackable microspheres in combination with imaging whole-mount mouse hearts, stained with fluorescent dyes for visualising microplastic particles, using two photon fluorescence and confocal microscopy could be explored.
7.4 Summary
The final chapter of this thesis assessed the use TIPS microspheres for delivery of iPSC-CM in vivo. The feasibility of delivering viable and trackable iPSC-CM on TIPS microspheres was validated in vitro, followed by in vivo assessment in an NSG mouse model. In summary:

- iPSCs were transduced using the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP, to express luciferase tisis x5 red 2A and eGFP, for cell sorting and BLI imaging.
- Transduced iPSC were trackable and retained iPSC phenotype, which were evaluated by morphology, FACS analysis and luciferase assay.
- Transduced iPSC were differentiated into eGFP+LUC+ iPSC-CM. The cardiac differentiation yield was lower than non-transduced control, but this was overcome by magnetic selection.
- To our acknowledge, this the first ever reported example of successfully transduced Gibco episomal iPSC for transgene expression of luciferase tisis x5 red 2A and eGFP. Differentiated eGFP+ Luc+ iPSC-CM were used as a trackable cell therapy in vivo.
- To examine whether iPSC-CM on TIPS microspheres could be effectively delivered by injection to the heart, cell survival post-injection was assessed in vitro. iPSC-CM on TIPS microspheres injected through a 23G capped syringe remained attached to TIPS microspheres up to 14 days post-injection and retained their beating phenotype.
- Transduced iPSC-CM laden TIPS microspheres were delivered into the left ventricular myocardium of 4-week-old male NSG mice via ultrasound guidance. Cardiac function was not adversely affected, however BLI signal showed a decrease in signal intensity over the 14-day time course. Histology confirmed the implantation of TIPS microspheres, but due to time limitations the results could not be further validated.

Overall, this chapter presents the final results of this thesis and a graphical summary of Chapter 7 is illustrated in Figure 7.21. Conclusive remarks and further work are discussed in Chapter 8.
Figure 7.21: Graphical summary of Chapter 7.
Chapter 8: Conclusion and future directions

The range of solutions available for CHF remain limited as current therapies fail to manage the progression of the disease. A heart transplant often becomes the only remaining solution.\textsuperscript{18} However, a heart transplant is not viable for all the patients that are affected by CHF due to the limited supply of available and compatible organs. Hence, there is a clear need for the development of a new therapies designed specifically to stop the progression of CHF.

There has been an increasing interest in the use of cell-based therapy to provide a solution for CHF. Over the past 20 years, cell-based therapies have been investigated for the regeneration of damaged heart tissue arising from ischaemic insults\textsuperscript{20}. To date, despite some studies showing limited beneficial effects of cell therapy (measured by improvement of ventricular function), clinical trial results have generally been disappointing with studies not meeting their primary outcome measures and the improvement of cardiac function has been minimal\textsuperscript{73}. Poor cell retention and engraftment is one of the main factors attributed to the response observed, with some studies reporting a 90% loss of cells within a few hours.

Cell therapy for cardiac regeneration is associated with numerous challenges. The ischaemic cardiac tissue presents a harsh environment for cell transplantation. The mechanical action of the heart also increases the risk of wash out and leakage of cells with every cardiac cycle. Additionally, adherent cells are maintained in suspension for hours prior to delivery, resulting in loss of cell viability.

Biomaterials have been used to improve cell delivery and survival; however, they are most often delivered in an invasive manner. After administration therapies are not further tracked, missing an opportunity to further optimise the therapy. Overall, the above-mentioned factors lead to a loss of potency and lack of efficacy. This study aimed to improve the delivery and engraftment of viable cells using TIPS microspheres to provide an implantable, biodegradable substrate for attachment and growth of iPSC-CM.

The strength of this project is reflected in the breadth of techniques utilised to investigate the hypothesis of the thesis towards the generation of novel findings. The findings presented in this thesis explore the holistic development of TIPS
microspheres as a novel intramyocardial delivery method for iPSC-CM, describing the optimisation of the microsphere formulation and the translation of experiments from *in vitro* to *in vivo* research models.

Chapter 3 described the fabrication and characterisation of TIPS microspheres, as well as the functionalization of the microspheres to enable iPSC attachment and maintenance of cell phenotype in xeno-free conditions. PLGA polymer composition, wetting solution composition and protein coating were optimised to determine a suitable TIPS microsphere formulation for iPSC attachment. 2% 7507 TIPS microsphere formulation, sieved to <250 µm and wetted in 5 µg/ml VTN-N HBSS solution was selected for further experiments. The wetting formulation was the first example of an animal component-free wetting solution used for processing TIPS microspheres. In addition to this, the wetting parameters measured were used to characterise the fabrication process and processing time. Chapter 3 presented the first ever reported attachment of human iPSC to TIPS microspheres, which could provide a platform for iPSC expansion and differentiation into non-cardiac cell types for different applications.

In Chapter 4, the compatibility of the microsphere formulations was assessed for minimally invasive delivery to the heart. TIPS microspheres were successfully formulated for minimally-invasive delivery. Preliminary studies suggested that acellular microspheres could be delivered to the top, mid and basal regions of the myocardium of a rat model using ultra-sound guidance, without negative effects on cardiac function. Additionally, a trackable microsphere formulation was sought to visualise the grafted material and be used to verify injection success, retention, and distribution of the microspheres. BaSO$_4$ loaded TIPS microsphere successfully produced CT trackable microspheres *in vitro* and *in vivo*. Although no cell therapy was applied in this study, the findings suggested that BaSO$_4$-loaded microspheres could be used as a novel tool for optimising delivery techniques and tracking persistence and distribution of implanted products.

Chapter 5 assessed the biocompatibility of the formulated TIPS microspheres with iPSC phenotype, as well the biomaterial-derived protection from anoikis. Firstly, iPSC growth kinetics were assessed by comparing cell expansion on TIPS microspheres versus control TC conditions. iPSC expanded on TIPS microspheres
and the expansion was comparable to the growth kinetics of iPSC cultured on TC conditions. iPSC attachment to VTN coated TIPS microspheres retained high alkaline phosphatase activity and expression of pluripotency markers SOX2 and OCT4. Interestingly, expression of extracellular pluripotent markers SSEA-4 and TRA-1-60 was reduced below 50% positive cells after interaction with VTN coated TIPS microspheres. This effect was dependent on protein coating and TIPS microsphere interaction. However, it was not translated from a reduction in pluripotent gene markers. Additionally, attachment of iPSC and iPSC-CM to TIPS microspheres in suspension culture indicated a protective phenotype against suspension-induced apoptosis and necrosis, suggesting protection from anoikis. Key tools were developed for the analysis of iPSC trilineage differentiation and of anoikis. These methodologies could be useful for any investigations assessing iPSC-based cell therapies.

Chapter 6 described the cardiac differentiation and enrichment strategies for the generation of TIPS microspheres cellularised with iPSC-CM. The cardiac phenotype was assessed by flow cytometry, confocal microscopy, and electrophysiology. The flow cytometry panel was designed to assess cardiac phenotype by the expression of cTNT and α-SMA cardiac markers and designed to complement the iPSC panel described in Chapter 5. iPSC were differentiated into a mixed iPSC-CM population using a commercially available kit and enriched by magnetic selection. iPSC differentiation into cardiomyocytes following attachment to the microspheres was also investigated. With the method devised, iPSC produced a low yield of cTNT and α-SMA expressing iPSC-CM when differentiated on TIPS microspheres. However, attachment of pre-differentiated iPSC-CM to TIPS microspheres was successful. iPSC-CM on TIPS microsphere retained target phenotype, as characterised by confocal microscopy and contractile behaviour. Optical mapping was used to assess iPSC-CM contractile functionality on the microsphere construct, which presented a novel application for calcium imaging. The technique could provide further insight into the role of engineered scaffolds in iPSC-CM functionality and maturation.

Finally, delivery of iPSC-CM laden TIPS microspheres was assessed in vivo. iPSC were transduced using the lentiviral vector pSEWFLAGx3-FLuc_tisisx5_red-2A-eGFP, to express luciferase tisis x5 red 2A and eGFP, for cell sorting and BLI imaging. Transduced iPSC retained their pluripotent phenotype and were
differentiated into eGFP+LUC+ iPSC-CM. The cardiac differentiation yield was lower than the non-transduced control, but this was overcome by metabolic selection. Transduced iPSC-CM laden TIPS microspheres were delivered into the left ventricular myocardium of immunocompromised mice via ultrasound guidance. Cardiac function was not adversely affected, however BLI signal showed a decrease in signal intensity over the 14-day time course. Histology confirmed the implantation of TIPS microspheres, but due to time limitations the results could not be further validated.

It will be informative to further validate the histological samples to determine iPSC-CM engraftment and survival, which BLI tracking might have missed. iPSC-CM cellularised TIPS microspheres would also allow for further development and analyses: for example, inclusion of a pro-survival cocktail might potentiate TIPS microsphere derived cell engraftment and survival rate. This approach would not only target anoikis, but also cell survival in an inflammatory environment. Pro-survival cocktails are generally composed of five pro-surviving factors: Matrigel, caspase inhibitor ZVAD-fmk, Bcl XL-derived peptide, IGF-1, pinacidil which opens mitoKATP, and a non-immunosuppressive dose of cyclosporine which acts on the mitochondrial permeability transition pore. Pro-survival cocktails have been shown to improve cell survival through anti-apoptotic effects and the formulation has been widely used in iPSC-CM and mixed cardiac cell therapies. The results could then be further validated in a pre-clinical model of CHF. The remaining questions associated with this thesis and the proposed future work to answer the questions are summarised below:
Gaps of knowledge

Chapter 3
Selection of PLGA TIPS microsphere for attachment of iPSC
1. More detailed material characterisation.
2. Why do microspheres shrink during wetting?
3. What is the degradation rate of 2% 7507 TIPS microspheres?

Chapter 4
Functionalization of TIPS microspheres for in vivo tracking
1. What was the frequency of off-target brain delivery of 20% BaSO4 loaded TIPS microspheres?
2. Can 20% BaSO4 loaded TIPS microspheres be made more biocompatible for pre-clinical tracking experiments?

Chapter 5
In vitro biological characterization of iPSC and cellularised TIPS microspheres
1. Can iPSC attachment efficiency to TIPS microspheres be improved?
2. Does TIPS microsphere interaction affect iPSC pluripotent phenotype? (i.e. by guiding iPSC transition into a trilineage differentiation progenitor)
3. Development of an assay specific for anoikis

Chapter 6
Characterisation of iPSC-CM differentiation
1. Can iPSC differentiate into iPSC-CM on TIPS microspheres using an alternative method?
2. Can iPSC-CM enrichment be improved?
3. Can iPSC-CM attachment efficiency to TIPS microspheres be improved?

Chapter 7
In vivo implantation of cellularised TIPS microspheres
1. Can iPSC-CM luminescence be improved?
2. Further validation of histological samples to determine cellular engraftment.
3. Will the inclusion of a pro-survival cocktail potentiate TIPS microsphere assisted cell engraftment and survival?

Proposed research plan

In vivo experiments
- Repeat in vivo experiment described in Chapter 7, with inclusion of a pro-survival cocktail to potentiate TIPS microsphere derived cell engraftment and survival rate in a healthy murine model.
- If the preliminary in vivo experiments suggest that TIPS microspheres improve cellular engraftment and survival, repeat the experiment in a preclinical murine model of CHF. LAD ligation followed by I/R.
- If the results obtained from the disease model show efficacy, translate the experiment into a bigger animal model, such as mini pigs.

TIPS microsphere characterisation
- Characterise microsphere topography and stiffness by atomic force microscopy.
- Shrinking of microsphere diameter might occur from collapse of internal microsphere porous structures, which could be investigated by cryo electron microscopy analysis of wetted samples.
- Assess microsphere degradation over 12 weeks in simulated in vivo conditions (37°C dynamic conditions).

TIPS microspheres processing
- Improve iPSC and iPSC-CM attachment efficiency to TIPS microspheres by optimising the static-dynamic attachment protocol parameters. Parameters include static dynamic timings, seeding-, microsphere-, and media-volumes.
- Investigate coating of TIPS microspheres with fibronectin to improve iPSC-CM attachment.

TIPS microspheres processing
- Supplement qPCR experiment with a human cell lineage identification profiler PCR array, to identify the mechanism of a TIPS microsphere-induced differentiation process.
- Repeat qPCR experiment, increasing n number and include additional markers.
- Develop an anoikis assay, investigating changes in integrin expression profiles.

Trackable microspheres
- Explore encapsulation of 20% BaSO4 loaded TIPS microspheres to improve biocompatibility for pre-clinical tracking experiments.
- Explore non-cardiac applications of trackable microspheres, i.e. therapeutic embolisation.

In vitro experiments (iPSC-CM)
- Assess cardiac differentiation of iPSC on TIPS microspheres using different iPSC lines. Cell lines that differentiate at higher confluence at the onset of differentiation, might differentiate on the microspheres.
- Single cell purification of transduced iPSC might improve iPSC-CM luminescence by reducing contamination of non-transduced cells.
Two types of biomaterials have been at the forefront of cardiac regeneration: hydrogels and cardiac patches. Injectable hydrogels have been designed with different base materials and cell combinations to target different or multiple aspects of cardiac regeneration. Notably, Gerbin et al. used a Delta-1 functionalised collagen based-hydrogel to deliver a subtherapeutic dose of hESC-CM into the infarcted rat myocardium\textsuperscript{397}. Transplantation of the hydrogel and hESC-CM significantly augmented heart function, measured by ECG fractional shortening, after 3 months of transplantation\textsuperscript{397}. Graft size and hESC-CM proliferation also increased through activated Notch signalling by presentation of Notch ligand, Delta-1\textsuperscript{397}.

Cell therapy delivery by cardiac patch implantation has shown to improve cell retention and functionalisation\textsuperscript{398,399}. Cardiac patches are typically composed of cells, bioactive molecules and biomaterials to fabricate the patch scaffold\textsuperscript{399}. Recent advances in therapeutic cardiac patches have been reviewed by Mei and Cheng\textsuperscript{399}. Importantly, Eschenhagen\textsuperscript{400–402}, Zimmerman\textsuperscript{400–402} and Sawa\textsuperscript{403–405} have been at the forefront of cardiac patch development, with the first in-patient trials of engineered iPSC-CM loaded cardiac patches (BioVAT-HF NCT04396899) for the treatment of CHF currently ongoing\textsuperscript{406}. However, patches often do not engraft, as they are frequently isolated from the host myocardium and separated by reactive epicardial fibrosis.

The TIPS microspheres bring benefits from both hydrogel and cardiac patch approaches; they are injectable, with robust surface attachment allowing for minimally invasive delivery, dispersal throughout the myocardium and potentially better engraftment into the tissue. Additionally, TIPS microspheres have the potential to be developed further to provide a freezable cell substrate for long term banking of hypo-immunogenic cells, unlike engineered heart tissues that need to prepared fresh. The microspheres could also be functionalised to encapsulate bioactive molecules, such as slow releasing survival or angiogenic factors to improve cardiac function.

In conclusion, this work demonstrates that TIPS microspheres offer a supporting matrix for culturing iPSC and iPSC derived cardiomyocytes \textit{in vitro} and when implanted \textit{in vivo} have the potential to be further developed into an injectable
biomaterial for cardiac regeneration. Overall, this work provides novel understanding of iPSC, iPSC-CM, and biomaterial assisted cell therapy. Despite some challenges, the advantages of using a biomaterial approach contributes to advancing the efficacy and design of cell therapies for future clinical applications.

Overall, the promise of cardiac rejuvenation remains unmet but has enabled the development of multiple research strategies towards cardiac regeneration. The knowledge of cardiogenesis and underlying regulatory pathways have been highly refined, but the field has also come to appreciate the physiological importance and therapeutic potential of other cell populations, such as the epicardium, the vasculature and the immune system. The field of cardiac regenerative medicine has seen developments in cell therapies, tissue engineering approaches, small molecule-driven cardiomyocyte regeneration, extracellular vesicle-derived therapeutics, gene/microRNA editing, direct reprogramming therapy and more. Whilst the first generation of cardiac regenerative therapies has taken a reductionist approach, focusing on single agents/approaches, it is likely that embracing the complex hallmarks of cardiac regeneration is required. The hallmarks include remuscularisation of the cardiac wall, electromechanical stability of remuscularised grafts, angiogenesis to provide a vascular network for implanted grafts, resolution of fibrosis and immunological balance to clear necrotic cells, resolve inflammation and allow regeneration. Hence, I envision the use of holistic approaches, combining small molecules, stem cells, bioengineering and gene editing, ultimately targeting the complexity of heart regeneration to achieve clinically relevant cardiac rejuvenation in the future.
Figure 9.1: Sinking of 2% 7507 <250 µm TIPS microspheres in 5-40% FBS wetting solutions at day (A) 0, (B) 1, (C) 2, (D) 3, and (ii) respective side views, showing floating microspheres.
Figure 9.2: Sinking of 2% 7507 <250 μm TIPS microspheres in 5 μg/ml VTN-N wetting solution at day (A) 0, (B) 4, (C) 7, (D) 10, (E) 14.
Figure 9.3: Sinking of 2% 7507 TIPS microspheres, sieved into size ranges, in 5 ug/ml VTN-N wetting solution at weeks (A) 2, (B) 3, (Ci) 5, and (ii) respective side view.
Figure 9.4: Wetting of 2% 7507 TIPS microspheres <250 µm, in 5% FBS and HBSS wetting solution at days (A) 0, (B) 1, (C) 2, and (D) 3 post-wetting. The samples were used for quantification of protein adsorption to the TIPS microspheres, by BCA assay.
Figure 9.5: Wetting of 2% 7507 TIPS microspheres <250 µm, in 10% FBS and HBSS wetting solution at days (A) 0, (B) 1, (C) 2, and (D) 3 post-wetting. The samples were used for quantification of protein adsorption to the TIPS microspheres, by BCA assay.
Figure 9.6: Wetting of 2% 7507 TIPS microspheres <250 µm, in 20% FBS and HBSS wetting solution at days (A) 0, (B) 1, (C) 2, and (D) 3 post-wetting. The samples were used for quantification of protein adsorption to the TIPS microspheres, by BCA assay.
Figure 9.7: Wetting of 2% 7507 TIPS microspheres <250 µm, in 5 µg/ml VTN-N and HBSS wetting solution at days (A) 1, (B) 2, (C) 4, (D) 6, (E) 11, (F) 12, (G) 14, and (H) 16 post-wetting. The samples were used for quantification of protein adsorption to the TIPS microspheres, by BCA assay.
Figure 9.8: Size range distribution of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm, (D) 425-500 µm, at (i) 3 days, (ii) 5 days and (iii) 7 days after wetting. n=100.
Figure 9.9: Circularity distribution of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm, (D) 425-500 µm, at (i) 3 days, (ii) 5 days and (iii) 7 days after wetting. n=100
Figure 9.10: Diameter distribution of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm, (D) 425-500 µm, after (i) 1 week and (ii) 2 weeks of simulated dynamic degradation. n=100.
Figure 9.11: Circularity distribution of TIPS microspheres (A) <250 µm, (B) 250-355 µm, (C) 355-425 µm, (D) 425-500 µm, after (i) 1 week and (ii) 2 weeks of simulated dynamic degradation. n=100.
Figure 9.12: Flow through of microspheres resuspended in HBSS through a 23G needle. Microspheres (A) <250, (B) 250-355, (C) 355-425 and (D) 425-500 µm diameter, were suspended in 5 ml of HBSS and loaded in (i) a syringe capped with a 23G needle. (ii) the suspension was extruded through the needle and flow through or obstruction at the neck of the needle was observed. Yellow arrows indicate microsphere obstructing the syringe.
Figure 9.13: Flow through of microspheres resuspended in 60% GRANUGEL™ through a (A) 23G and (B) 25 G needle. Microspheres <250 µm diameter, were suspended in 1 ml 60% GRANUGEL™ formulation, loaded in a syringe capped with a 23G or 25G needle. The solution was extruded through the syringe and obstruction at the neck of the needle was observed in both conditions. Majority of the microspheres managed to flow through the 23G needle, but none flowed through the 25G needle. Yellow arrows indicate microsphere obstruction at the neck of the needle.
Figure 9.14: Characterization of TIPS microspheres morphological parameters with Morphologie G3: (A) diameter, (B) circularity, and (C) aspect ratio. The respective mean values are marked in red. n= 3 separate production batches, n1:1770 , n2:1907 and n3:1879 microspheres.
Figure 9.15: (A) MIP reconstruction of whole animal CT scan at day 1 post-injection, with corresponding (B) sagittal, (C) coronal and (D) transversal scans.
Figure 9.16: (A) MIP reconstruction of *ex vivo* CT scan, with corresponding (B) sagittal, (C) coronal and (D) transversal scans.
Figure 9.17: Sample ROIs for quantification of heart volume (green) and the contrast signal from BaSO$_4$ loaded TIPS microspheres signal (yellow). (A) Sagittal, (B) coronal and (C) transverse slice.
Figure 9.18: Sample ROIs for quantification of organ volume and the contrast signal from BaSO₄ loaded TIPS microspheres signal. (i) Sagittal and (ii) coronal slice.
Figure 9.19: Contrast signal of BaSO4 loaded TIPS microspheres in ex-vivo organs, (A-E) 6 days post-implantation in rat 1-5 and (F) 0 days post-implantation in rat 6.
Figure 9.20: Determination of pluripotent qPCR primer amplification efficiency.
Figure 9.21: Determination of mesoderm qPCR primer amplification efficiency.
Figure 9.22: Determination of endoderm qPCR primer amplification efficiency.
Figure 9.23: Determination of ectoderm qPCR primer amplification efficiency.

A. ALDH1A0

\[ y = -2.462x + 30.314 \]

\[ R^2 = 0.9694 \]

Efficiency: 2.55

B. COL1A1

\[ y = -2.9114x + 27.22 \]

\[ R^2 = 0.9876 \]

Efficiency: 2.21

C. PAX6

\[ y = -3.3376x + 26.384 \]

\[ R^2 = 0.9859 \]

Efficiency: 1.99

D. DCX1

\[ y = -3.245x + 30.083 \]

\[ R^2 = 0.9575 \]

Efficiency: 2.03
Figure 9.24: Determination of housekeeping qPCR primer amplification efficiency.
Figure 9.25: Light microscopy images of TIPS microspheres samples used for characterisation of anoikis-dependent apoptosis.
Figure 9.26: Light microscopy images of control and suspension iPSC samples used for characterisation of anoikis-dependent apoptosis.
Figure 9.27: Light microscopy images of control and suspension iPSC-CM samples used for characterisation of anoikis-dependent apoptosis.
Figure 9.28: Gating strategy (A) used for the separation of positive and negative iPSC-CM populations stained with dilutions of cTNT AF647 antibody at (B) 1:50, (C) 1:100, (D) 1:200, and (E) 1:1000.
Figure 9.29: Titration of cTNT AF647 on iPSC-CM. (A) measurement of MFI and SD of the negative and positive populations, (B) formula, (C) calculated, and (D) plotted stain index values. Panel B reproduced from BioRad.
**A. Episomal iPSC, 5 μM CHIR**

<table>
<thead>
<tr>
<th>Days in differentiation protocol</th>
<th>Colony confluence</th>
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<tbody>
<tr>
<td>0</td>
<td>30%</td>
</tr>
<tr>
<td>2</td>
<td>50%</td>
</tr>
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<td>4</td>
<td>70%</td>
</tr>
<tr>
<td>6</td>
<td>90%</td>
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Figure 9.30: Differentiation of iPSC into iPSC-CM using the Burridge *et al.* differentiation protocol with 5 μM CHIR99021 at day 0-2. Light microscopy images of episomal iPSC 4 days after seeding, at 30%, 50%, 70% and 90% colony confluence. Confluence measurements were performed by visual estimation. The colonies were differentiated into iPSC-CM for 10 days. Scale bar represents 100 μm.
### B. Episomal iPSC, 6 µM CHIR

<table>
<thead>
<tr>
<th>Colony confluence</th>
<th>30%</th>
<th>50%</th>
<th>70%</th>
<th>90%</th>
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<tr>
<td>0</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
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<td>2</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
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<td>4</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
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<td>6</td>
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<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
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<tr>
<td>10</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
<td>![Image](72x236 to 525x770)</td>
</tr>
</tbody>
</table>

Figure 9.31: Differentiation of iPSC into iPSC-CM using the Burridge *et al.* differentiation protocol with 6 µM CHIR99021 at day 0-2. Light microscopy images of episomal iPSC 4 days after seeding, at 30%, 50%, 70% and 90% colony confluence. Confluence measurements were performed by visual estimation. The colonies were differentiated into iPSC-CM for 10 days. Scale bar represents 100 µm.
Figure 9.32: Differentiation of iPSC into iPSC-CM using the Burridge et al. differentiation protocol with 7 µM CHIR99021 at day 0-2. Light microscopy images of episomal iPSC 4 days after seeding, at 30%, 50%, 70% and 90% colony confluence. Confluence measurements were performed by visual estimation. The colonies were differentiated into iPSC-CM for 10 days. Scale bar represents 100 µm.
Figure 9.33: Differentiation of episomal iPSC into iPSC-CM using the commercial differentiation kit from Thermo Fisher. Light microscopy images of episomal iPSC 4 days after seeding, at 30%, 50%, 70% and 90% colony confluence. Confluence measurements were performed by visual estimation. The colonies were differentiated into iPSC-CM for 8 days. Contractile phenotype was observed by the end of the protocol. Scale bar represents 100 µm.
Figure 9.34: Attachment of iPSC onto TIPS microspheres and differentiation into iPSC-CM using the commercial differentiation kit from Thermo Fisher. After attachment, the cells continued cardiac differentiation up to day 20. Scale bar represents 100 µm.
Figure 9.35: Attachment of partially differentiated (day 2 differentiation) iPSC-CM onto TIPS microspheres and differentiation into iPSC-CM using the commercial differentiation kit from Thermo Fisher. After attachment, the cells continued cardiac differentiation up to day 20. Scale bar represents 100 µm.
Figure 9.36: Attachment of partially differentiated (day 4 differentiation) iPSC-CM onto TIPS microspheres and differentiation into iPSC-CM using the commercial differentiation kit from Thermo Fisher. After attachment, the cells continued cardiac differentiation up to day 20. Scale bar represents 100 µm.
Figure 9.37: Attachment of differentiated (day 6 differentiation) iPSC-CM onto TIPS microspheres. After attachment, the cells were cultured in cardiac maintenance media up to day 20. Scale bar represents 100 µm.
Figure 9.38: Attachment of differentiated (day 18 differentiation) iPSC-CM onto TIPS microspheres. After attachment, the cells were cultured in cardiac maintenance media up to day 20. Scale bar represents 100 µm.
Figure 9.39: Magnetically enriched iPSC-CM monolayer utilised for optical mapping. The enriched iPSC-CM were replated as a confluent monolayer on VTN-N coated 35mm tissue culture dish. Light microscopy images show the formation of the monolayer from (A) 24 hours, (B) 48 hours and (C) 7 days post-seeding. Scale bar represents 100 µm.
<table>
<thead>
<tr>
<th>Model</th>
<th>CHF stimulus</th>
<th>Species</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surgical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LV pressure overload</td>
<td>TAC</td>
<td>Rat and mouse</td>
<td>Reliable model to induce cardiac hypertrophy and HF.</td>
<td>The acute increase in afterload does not reflect the gradual progression of arterial hypertension and aortic valve stenosis in patients.</td>
</tr>
<tr>
<td>Ascending aortic constriction</td>
<td></td>
<td>Rat</td>
<td>Gradual onset of pressure overload mimics the gradual progression of arterial hypertension in patients.</td>
<td>Limited relevance to human disease as pressure overload is induced in young animals, whereas arterial hypertension is primarily observed in elderly patients.</td>
</tr>
<tr>
<td>Temporary LV pressure overload</td>
<td>TAC+ removal of stenosis</td>
<td>Mouse</td>
<td>Reliable model of cardiac hypertrophy followed by removal of stressor to study reverse cardiac remodelling.</td>
<td>Two surgeries are required and the removal of sutures/ clips are technically challenging techniques.</td>
</tr>
<tr>
<td>MI</td>
<td>LAD ligation</td>
<td>Rat and mouse</td>
<td>Reliable model to induce tissue damage and HF.</td>
<td>Model does not reflect the clinical setting with reperfusion of the occluded vessel during coronary angiography, performed after an acute MI.</td>
</tr>
<tr>
<td>Ischaemia/reperfusion injury</td>
<td>Temporary LAD ligation</td>
<td>Rat and mouse</td>
<td>Mimics the clinical scenario with reperfusion of the occluded vessel during coronary angiography performed after an acute MI.</td>
<td>Surgery is more time consuming and more complex than placement of permanent LAD ligation.</td>
</tr>
<tr>
<td>Pressure overload and MI</td>
<td>TAC+ LAD ligation</td>
<td>Mouse</td>
<td>Model mimics the relevant co-morbidities of arterial hypertension and ischaemic heart disease. Gradual and predictable progression of HF.</td>
<td>Acute increase in afterload does not mimic the clinical setting of arterial hypertension and aortic valve stenosis in patients.</td>
</tr>
<tr>
<td>Ascending aortic constriction+ LAD ligation</td>
<td>Rat</td>
<td>Same as for mouse model of TAC + LAD ligation.</td>
<td>Same as for mouse model of TAC + LAD ligation.</td>
<td>Same as for mouse model of TAC + LAD ligation.</td>
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<tr>
<td>Volume overload</td>
<td>Abdominal aortic constriction + LAD ligation</td>
<td>Rat</td>
<td>Same as for mouse model of TAC + LAD ligation.</td>
<td>Same as for mouse model of TAC + LAD ligation.</td>
</tr>
<tr>
<td>-----------------</td>
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<td>-----------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Aorto-caval fistula (shunt)</td>
<td>Rat and mouse</td>
<td>Model of chronic volume overload as observed in patients with mitral valve regurgitation. Reproducible model of volume overload induced HF.</td>
<td>Shunt creates an artificial mix of arterial with venous blood.</td>
<td></td>
</tr>
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</table>

**Drug Induced**

<table>
<thead>
<tr>
<th>Toxic cardiomyopathy</th>
<th>Doxorubicin</th>
<th>Rat and mouse</th>
<th>Potent stimulus to induce dilated Cardiomyopathy.</th>
<th>Systemic toxic effects, especially on bone marrow cells, and gastrointestinal system.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>Rat and mouse</td>
<td>Potent stimulus to induce cardiac hypertrophy. Drug is easy to administer (i.p. injection or osmotic mini pump).</td>
<td>Chronic activation of adrenergic signalling is only one contributing factor to the development of HF in patients</td>
<td></td>
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<tr>
<td>Monocrotaline</td>
<td>Rat</td>
<td>Model of predominantly RV hypertrophy and RV failure.</td>
<td>Toxicity on other organ systems, i.e. pulmonary and kidney injury.</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>Rat</td>
<td></td>
<td>Non-specific side effects and toxicity on other organ systems, especially liver.</td>
<td></td>
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**Genetic**

<table>
<thead>
<tr>
<th>Spontaneously hypertensive rats</th>
<th>Inbred strain of Wistar-Kyoto rats with hypertension</th>
<th>Rat</th>
<th>Slow progression of hypertension and HF development as observed in patients.</th>
<th>High housing costs based on the slow progression of hypertension and HF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl salt-sensitive rat</td>
<td>Inbred strain of Sprague-Dawley rats, that are susceptible to hypertension following a high salt diet.</td>
<td>Rat</td>
<td>Induction of hypertension and HF by high-salt diet feeding without additional surgery. Slow progression of hypertension and HF development as observed in patients.</td>
<td>High housing costs based on the slow progression of hypertension and HF.</td>
</tr>
</tbody>
</table>

Table 9.1: Characteristics of small animal models of CHF. Adapted from Riehle & Bauersachs. Intraperitoneal (i.p.), transverse aortic constriction (TAC), myocardial ischaemia (MI), heart failure (HF), left ventricle (LV), left anterior descending artery (LAD).
Figure 9.40: Representative ROIs used to quantify transduced iPSC-CM luminescence in BLI images. The ROIs used to quantify luminescence from iPSC-CM delivered in (A) suspension and (B) attached to TIPS microspheres. Images acquired 2 hours post-injection.
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