Universidade de Aveiro Departamento de Química
Ano 2021

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Catarina de Almeida Custódio, Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ana Sofia Silva, Investigadora Júnior do Departamento de Química da Universidade de Aveiro.

FCT Fundação para a Ciência e a Tecnologia
erc European Research Council
ciceco aveiro institute of materials
European Commission
Horizon 2020 European Union funding for Research & Innovation

Aos meus pais e irmã

o júri

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agradecimentos

Primeiramente gostaria de agradecer ao Prof. João Mano pela oportunidade que me deu em poder realizar este projeto no COMPASS Research Group.

Agradeço à Dra. Catarina Custódio pelo apoio e orientação que me deu sempre e também pela enorme paciência. Também um grande obrigado a ti Dra. Sofia que me apoiaste durante este meu percurso.

Obrigado a todos os membros do COMPASS por toda a ajuda que deram e companheirismo, e especialmente a quem contribuiu diretamente para que eu conseguisse concluir este projeto e a todos os que me deram motivação no momento certo.
Deixo aqui um agradecimento muito especial à Sara que me ajudou e motivou sempre até ao fim, és sem dúvida uma pessoa que terei sempre grande consideração e estima.

Um grande abraço a todos os meus amigos que longe ou perto de mim me apoiaram. Agradeço com especial carinho aos que me ouviram lamentar vezes sem conta até ao fim desta luta. Um grande obrigado a vocês por acreditarem sempre que era possível mesmo quando eu não acreditava.

E claro, o maior agradecimento vai para as pessoas mais importantes da minha vida. Obrigado pai e mãe por me apoiarem independentemente de tudo o que aconteça, sei que estão e vão estar sempre dispostos a ajudar-me e é a sensação de conforto mais incrível que tenho. Sem vocês nunca teria conseguido chegar onde cheguei. Maninha, obviamente que não podias faltar nos agradecimentos, sabes como te adoro. Muito obrigado por teres sempre uma palavra de carinho para me dar.

Este projeto além de ter sido uma experiência profissional extremamente desafiante, foi sem dúvida uma soma constante de lições pessoais que levo comigo para o resto da vida. Obrigado a todos os que fizeram parte direta ou indiretamente desta etapa.

The present work was financially supported by the projects Beat (PTDC/BTM-MAT/30869/2017) and PROMENADE (PTDC/BTM-MAT/29830/2017), and by the European Research Council (ERC) for project ATLAS (ERC-2014-ADG-669858). The work was also partially supported by the European Union (EU) Horizon 2020 for the project InterLynk, Grant agreement: H2020-NMBP-TR-IND-2020, Project ID: 953169.

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, FCT Ref. UID/CTM/50011/2019, financed by national funds through the FCT/MCTES.

palavras-chave Engenharia de tecidos, *patches* cardíacos, hidrogéis, PLMA, micropilares, superfícies micropadronizadas, microfabricação

resumo

As doenças cardíacas são uma das principais causas de morte em todo o mundo. O enfarte do miocárdio é um dos problemas mais graves, levando à destruição de tecido cardíaco funcional e colocando os doentes em risco constante de insuficiência cardíaca.

As terapias farmacológicas convencionais ou transplantes de órgãos não são muitas vezes eficientes e associadas a múltiplas limitações, pelo que a procura por outras alternativas é uma necessidade. A engenharia de tecidos surgiu como uma alternativa para produzir estruturas que potenciem a regeneração cardíaca ou o desenvolvimento de microtecidos para o teste de fármacos *in vitro*. A engenharia de tecidos combina tipicamente biomateriais, células e sinais bioquímicos. A estrutura 3D ideal para um *patch* cardíaco deve combinar várias propriedades estruturais e funcionais que devem corresponder às do miocárdio nativo. Os hidrogéis derivados de lisados de plaquetas humanas metacrilatados (PLMA) foram recentemente reportados como um biomaterial de base humana com propriedades mecânicas adaptáveis que suportam a cultura de células humanas. Neste projeto de tese, foram desenvolvidos hidrogéis micropadronizados à base de PLMA. Para tal, os PL foram quimicamente modificados com grupos metacrílicos, tornando-se assim foto-reticuláveis. Posteriormente, o polidimetilsiloxano (PDMS) foi utilizado para produzir estruturas micropadronizadas com micropoços para serem utilizados como contra-moldes. Foram feitos dois padrões diferentes de micropilares - hexagonais e quadrados - com uma estrutura bem definida. A caracterização mecânica demonstrou que os hidrogéis de PLMA micropadronizados têm propriedades mecânicas ajustáveis dependentes da concentração de polímero usado na solução precursora. Foram realizados ensaios biológicos para compreender a resposta das células à topografia e os resultados mostraram que os hidrogéis de PLMA suportam a adesão e proliferação tanto das células endoteliais da veia umbilical humana (HUVECs) como dos cardiomioblastos derivados do tecido embrionário do miocárdio de rato (H9c2(2-1)).

Neste trabalho fomos capazes de produzir hidrogéis micropadronizados à base de PLMA com propriedades biomecânicas adequadas, permitindo uma boa adesão celular, proliferação e pré-formação de um microtecido com potencial comunicação entre células no *scaffold*.

keywords Tissue engineering, cardiac patches, hydrogels, PLMA, micropillars, micropatterned surfaces, microfabrication

abstract

Heart diseases are a major cause of dead worldwide. Myocardial infarction is one of the most serious problems, leading to the destruction of functional heart tissue and putting patients at constant risk of heart failure.

Conventional pharmacologic therapies or organ transplantation are often not efficient and associated with multiple limitations, and therefore the search for other alternatives is a need. Tissue engineering (TE) has emerged as an alternative to produce structures that potentiate cardiac regeneration or microtissues development for *in vitro* drug screening. TE typically combines biomaterials, cells, and biochemical signals. The ideal 3D structure for a cardiac patch must combine several structural and functional properties that should match the existing in the native myocardium. Human methacryloyl platelet lysates (PLMA)-derived hydrogels have recently been reported as a human based biomaterial with tuneable mechanical properties that support human cell culture. In this thesis project, micropatterned PLMA-based hydrogels were developed. To do so, PLs were chemically modified with methacryloyl groups, thus becoming photocrosslinkable. Afterwards, polydimethylsiloxane (PDMS) was used to produce micropatterned structures with microwells to be used as counter molds. Two different micropillars patterns were made - hexagonal and square. Mechanical characterization shows that micropatterned PLMA hydrogels have mechanical properties dependent on the concentration of the polymer present in the precursor solution. Biological assays were performed in order to understand cells response to topography and results showed that PLMA-hydrogels support the adhesion and proliferation of both human umbilical vein endothelial cells (HUVECs) and myoblasts derived from embryonic myocardium rat tissue (H9c2(2-1)).

In this work we were capable to produce micropatterned PLMA-hydrogels which have suitable biomechanical properties, allowing a good cell adhesion, proliferation, and pre-formation of a microtissue with potential crosstalk between cells at the scaffold.

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List of abbreviations

2D	two-dimensional
3D	three-dimensional
ASC	adipose-derived stem cell
AVC	atrioventricular canal
BM	bone marrow
BM-MSC	bone marrow-derived mesenchymal stem cell
CM	cardiomyocyte
dECM	decellularized extracellular matrix
HUVEC	human umbilical vein endothelial cell
EC	endothelial cell
ECM	extracellular matrix
EMT	endothelial-to-mesenchymal transition
ESC	embryonic stem cell
GF	growth factor
GNR	gold nanorod
H9c2(2-1)	embryonic myocardium rat tissue
HA	hyaluronic acid
hESC	human embryonic stem cell
hESC-CM	human embryonic stem cell-derived cardiomyocyte
HF	heart failure
hiPSC	human induced pluripotent stem cell
hiPSC-CM	human induced pluripotent stem cell-derived cardiomyocyte
hiPSC-EC	human induced pluripotent stem cell-derived endothelial cell
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
LV	left ventricle
MeTro	methacrylated tropoelastin
MI	myocardial infarction
MSC	mesenchymal stem cell

OFT	proximal outflow tract
PCL	polycaprolactone
PEG	poly(ethylene glycol)
PEGDA	poly(ethylene glycol) diacrylate
PEO	proepicardial organ
PGS	polyglycerol sebacate
PL	platelet lysate
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic) acid
PLMA	methacryloyl platelet lysates
PMMA	poly(methyl methacrylate)
PU	polyurethane
ROS	reactive oxygen species
SMC	smooth muscle cell
TE	tissue engineering
TLR	Toll-like receptor
UV	ultraviolet
VEGF	vascular endothelial growth factor

Chapter I

Background

Background

Cardiovascular diseases are increasing all over the world, especially in developed countries.[1] In 2019, according to the World Health Organization, about 32% of deaths in the world have heart-related diseases as the main cause. This percentage corresponds to 17.9 million people, 85% of which were due to heart attack and stroke.[2] In Portugal, about 6.4% of total mortality in 2019 is represented by ischemic heart disease, and even though there has been a significant decrease in deaths compared to the previous year, the diseases by acute myocardial infarction (MI) correspond to 3.8% of national population death.[3]

MI is universally defined as a pathology associated with the cell death in the myocardium due to prolonged ischemia. This decrease in blood flow through the coronary arteries, the vessels that supply blood to the heart, is generally caused by the presence of fatty plaques inside them.[4] This way, the injured myocardium area is unable to transmit electrical signals or contract properly, putting patients at risk of subsequent heart failure (HF).[5]

Over the years, tissue engineering and regenerative medicine (TERM) approaches have been developed by researchers thus representing a scientific field which combines cells, biomimetic matrices and signalling factors to replicate human body tissues for their replacement or restoration, allowing the maintenance of normal biological functions.[6]

Cell-based therapies for cardiac diseases emerged as interesting alternatives to conventional pharmacological treatments and heart transplantation allowing the repair of irreversible negative changes in the biomolecular environment and the mechanical properties of the damaged myocardial tissue area taking advantage of its intrinsic regenerative capability.[7] The use of stem cells such as bone marrow-derived mesenchymal stem cells (BM-MSCs) due to their contribution to angiogenesis, neovascularisation, cell survival and even inhibition of proliferation of cardiac fibroblasts, thus reducing fibrosis, have led to numerous advantages for this type of therapy.[8,9] Several studies also refer the contribution in cardiac regeneration of human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) or human embryonic stem cells (hESCs) as well as human induced pluripotent stem cells (hiPSCs) and stem cells, especially BM-MSCs, as previously mentioned, and adipose-derived stem cells (ASCs) due to their identical characteristics to primary cardiomyocytes (CM) of the heart.[6,10,11]

Cell co-culture brings the possibility of creating interactions between them and combining properties such as efficient revascularization by endothelial cells (ECs) allowing the rapid induction of CM proliferation, when approaching the affected area.[12]

Cardiac regeneration studies demonstrate that cells grown in a 3D environment have better physiological characteristics than when cell cultures were performed at 2D biomaterials, allowing a closer mimicry of native myocardial tissue.

Surface micropatterning techniques can be used to create novel 3D-platforms from biomaterials, promoting directional properties and enabling the mimicry of the tissue's natural anisotropy.[13,14]

The aim of this master's thesis is to evaluate the use of methacryloyl platelet lysates (PLMA)-based hydrogels as potential micropatterned membranes for the regeneration of injured cardiac tissue after MI. This cardiac patch model is based on photopolymerization PLMA to produce hydrogels with a pattern of micropillars. Afterwards, such micropatterned PLMA hydrogels will be used to understand how cells like ECs or cardiomyoblasts respond to the specific topographies proposed, perhaps allowing to control their alignment and orientation. These platforms are intended to mimic the cardiac tissue so that, on one hand, they must have the mechanical and physiological properties close to the native tissue, and on the other hand, they must be able to support cell adhesion and proliferation.

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Chapter II

Tissue engineering strategies for cardiac regeneration

Tissue engineering strategies for cardiac regeneration

Abstract

Heart disease has proven to be a global problem that needs to be addressed by the scientific community. Myocardial infarction (MI) is one of the most serious problems, leading to the destruction of heart tissue, putting patients at constant risk of heart failure.

Conventional pharmacologic therapies or organ transplantation are often not efficient and associated with multiple limitations, and so the search for other alternatives is a need. Tissue engineering (TE) has emerged as an alternative through the production of structures with potential for application in cardiac regeneration.

TE combines cells and biomaterials to create bioengineered constructs with efficient mechanical and biochemical properties for *in vitro* or *in vivo* applications, allowing the construction of microenvironments that mimic myocardial tissue. In this review we give a brief overview of the natural healing process of the heart post MI. Subsequently are presented and discussed the biomaterials and processing techniques that have drawn the most attention in the field of cardiac repair. Finally, are discussed in more detail microfabrication approaches to engineer cardiac patches and their effect in cell function.

1. Introduction

Over the years, tissue engineering (TE) has developed clever strategies to solve many health-related problems that directly affect the human body. Many innovative methods used for tissue regeneration involve materials that are defined as "bioactive" which can bind to living organs forming a robust and chemically stable interface. Such materials are commonly referred to as biomaterials.[1] Biomaterials are expanding, being modified to develop more complex systems with more defined physicochemical properties.[2]

Cardiac pathologies are a leading cause of concern worldwide with these pathologies having a high fatality rate.[3] Clinical micro sequels of problems associated with myocardial infarction (MI) include a scar tissue in the heart, after natural remodelling, is unable to transmit electrical signals or to contract properly, putting patients at risk of subsequent heart failure (HF).[4] Other associated symptoms are: interruption of blood flow, plaque

rupture, coronary artery thrombosis or coronary occlusion, all of which also interfere negatively with the organism's immunity.[5]

To meet up these challenges, it is necessary to find the best therapies to treat the heart defect that appears after the infarction.[6] TE combines cells, biomaterials, and biochemical cues to develop substitute tissues and/or promote endogenous regeneration. Natural or synthetic derived materials have been using in TE applications. Hydrogels, due to their compositional and structural similarities to the natural extracellular matrix (ECM), have been in focus for many applications in tissue regeneration and drug delivery systems.[7] The dynamic cardiac environment requires the use of biomaterials that are soft but tough, in order to withstand the repetitive forces associated with the cardiac cycle without losing mechanical integrity. The ideal material for cardiac repair should tolerate cyclic loading forces for the resulting construct to function properly.[8,9]

An important part of the endogenous process of cardiac repair it is promoted by cells that are recruited to the site of injury. The ideal TE cardiac strategy must take into account this physiological response of the heart while providing an effective physical support with the ability to support efficient cell attachment and proliferation, that lead to the formation of functional cardiac tissue. In this review we present a brief description of the endogenous events that follow a myocardial infarction and the main strategies in TE that have been explored to help the natural process of cardiac repair. We also brief the biomaterials that have been proposed for cardiac TE with a particular focus in cardiac patches, highlight different microfabrication strategies that have been described improve their functionality and efficacy.

2. Heart natural regeneration post-MI

The heart is a muscular organ, which is responsible for pumping blood throughout the entire human body.[10] Heart has three different tissue layers on both the right (RV) and left (LV) ventricles: endocardium, myocardium, and epicardium.[11] The epicardium is the outer layer of the heart. The inner region of the myocardium is connected to this layer as well as the serous layer of the pericardium. The epicardium nourishes the subcutaneous myocardium with trophic factors during the development of the heart, contributing directly to the cardiac fibroblasts and giving rise to coronary vasculature. Other

functions are reused during responses to epicardial lesions through the reactivation and induction of certain embryonic markers.[12]

Epicardium derives from the proepicardial organ (PEO), a kind of cluster of cells, which after expanding allows cells to migrate from this structure to populate the surface of the heart and form the epicardium proper.[13]

The endocardium can be found in the inner layer of all four cardiac chambers and, through an endothelial-to-mesenchymal transition (EMT) process, forms the cardiac cushion mesenchyme in the atrioventricular canal (AVC) and proximal outflow tract (OFT).[14] The primary composition of endocardium consists of endothelial cells (ECs) and it is believed to control itself and the myocardium through the distribution of action potentials by Purkinje fibres within the heart muscle.[15] The layer of the heart between the two layers above mentioned (epicardium and endocardium) is called myocardium, which provides support to the heart chambers. Hence, it is a very complex tissue with multiple and highly interconnected lengthy scales in a well-defined structural and functional hierarchy. Myocardium helps heart in the contraction and relaxation of the heart walls, allowing for blood to pass between the chambers, as well as in the conduction of electrostimulation through its own tissues and epicardium. Nevertheless, the basic functional unit for contraction is regulated by the actin–myosin cross-bridge cycling mechanism.[16] The myocardium is mainly composed by cardiomyocytes (CMs) and fibroblasts but also contains ECs and mesenchymal stem cells (MSCs). CMs remains stable over the human lifespan, whereas ECs and MSCs.[17] Thus, during heart growth, CMs always represent a constant number, while the number of ECs and MSCs expands.[17]

Although the myocardium has distinct physiological, mechanical, microstructural, and electrical characteristics, after infarction it becomes fragile and at any time HF may occur. This happens since when the myocardium fails, some problems appear such as the myocardial tissue becoming too stiff and thus not allowing enough blood to fill the ventricles during diastole. Consequently, everything is further dilated and not exactly contracted during systole to meet the requirement of fractional ejection or presenting a regional malfunction due to acute injury from lack of oxygen and infarction.[16]

Acute MI also causes vascular disintegration and affects the capillaries due to massive lesions in the coronary microcirculation, which leads to inflammation process during which deaths of CMs occur.[6]

2.1. Cellular recruitment in myocardial ischaemia post-MI

When a MI occurs, besides the interaction of the cells, which come into action to obtain the most effective response to regenerate the injured tissue, there is also a more internal response which corresponds to the activation of specific genes which corresponds to each part, as observed in a study for the epicardium.[12] Finally, these migratory cells differ to give a variety of cells such as cardiac fibroblasts, coronary smooth muscle cells (SMCs), ECs, and CMs. The understanding of these cellular and molecular mechanisms is also important for the diagnosis of patients who have suffered a MI. In fact, cardiac enzymes present themselves as good biomarkers of MI prediction and their detection, which is easy and fast, allows an efficient prognosis in the perception of the severity of MI in a clinical trial. However, these immune biomarkers can often be masked in patients with pre-existing immune diseases.[18] To sum up, there are multiple overlapping stages of heart tissue repair (see figure 1) which include: inflammation, proliferation, and remodelling.[19]

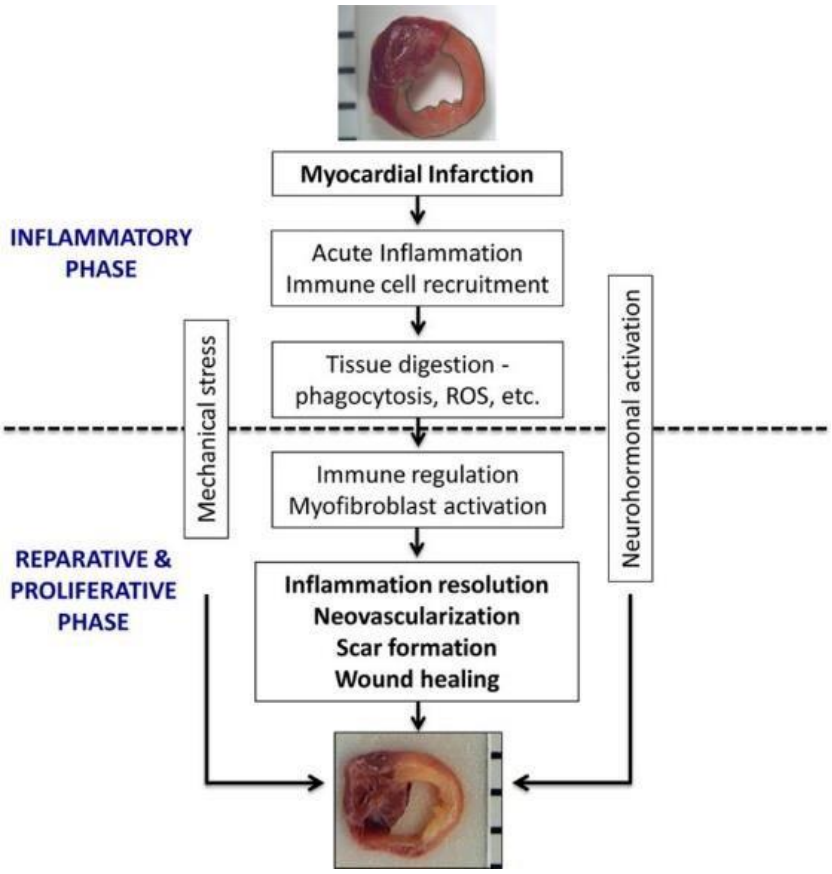

Figure II.1. Schematic illustration of biphasic nature of cardiac repair after myocardial infarction. ROS refers to "reactive oxygen species". [Adapted from "Circulation Research"][20]

2.1.1. Inflammation phase

MI results in cell death, triggering an inflammatory response, leading to scarring. This first phase is mediated by the inborn immune system. Platelets act soon leading to the prevention of bleeding due to injury to heart tissue. There is an aggregation of platelets which at the affected site leads to the formation of a fibrin-rich matrix. Then, it occurs a release of not only of growth factors (GFs), such as platelet-derived GF and platelet factor 4 that help in the repair process, but also platelet activation factors, thus stimulating the influx and adhesion of neutrophilic granulocytes to the site of injury.[21]

The neutrophils infiltration is the first process that leads to the recruitment of cellular debris such as cytokines and pro-inflammatory chemokines, including interleukins of necrotic and apoptotic cells.[19] The recruitment of neutrophils is activated by reactive oxygen species (ROS) produced by activated CMs and ECs. The ROS include hydrogen peroxides, superoxide anions and hydroxide radicals that are formed due to incomplete reduction of molecular oxygen, and activate other molecular components that cooperate in neutrophil recruitment.[22]

Besides neutrophils, which secrete proteolytic enzymes that clear the infarct from dead cells and debris, there are other phagocytes that are recruited, namely the monocytes, which also act to directly remove necrotic and apoptotic cells.[21] Finally, both monocytes and macrophages secrete GFs that can promote angiogenesis, specifically by focusing on and activating myofibroblasts. Myofibroblasts secrete the ECM and accumulate in the first week after a heart attack. These cells are critical for scar formation and prevention of cardiac dilation. However, too much matrix deposition, particularly in areas remote from the infarct, can also lead to HF. Therefore, this phase requires a careful and efficient immune response that does not allow excessive inflammation.[23]

Furthermore, the contribution of specific intracellular components that are released by necrotic CMs, which are detected by innate immune cells activated at the entrance of the tissue is crucial. The most prominent pathways, through which the innate immune system initiates an inflammatory response after infarction include the Toll-like receptor (TLR) which is a mediated pathway, the complement cascade, and the previously mentioned ROS.[21]

2.1.2. Proliferation phase

Repair continues, now moving on to the proliferative phase where the macrophage polarisation moves into the anti-inflammatory phenotype of M2 macrophages, promoting myofibroblast and vascular cell infiltration.[19] M2 macrophages, also called alternatively activated macrophages, are immune cells with high phenotypic heterogenicity and govern functions at the interface of immunity, tissue homeostasis, metabolism and endocrine signalling.[24]

At this stage, there is a cooperation of neutrophils, mononuclear cells, ECs and pericytes. Short-term neutrophils become apoptotic and release mediators that suppress neutrophil recruitment. Furthermore, there is an accumulation of mast cells during proliferation and cell fibrosis. Mast cells participate in the regulation of fibrosis by secreting compounds which contribute to the remodelling of damaged heart tissue. However, the exact role of mast cells in the process of cardiac inflammation is still under investigation.[21]

Thus, all processes at this stage not only lead to the formation of highly vascularized granulation tissue but also create a good pro-inflammatory environment allowing for better tissue repair.

2.1.3. Remodelling phase

In the remodelling phase, the formation of the scar is finalised after the proliferation phase. This process is followed by tissue maturation caused by the proliferation of ECs, thus forming an extensive microvascular network.[21] After MI, a continuous activation response of fibroblasts such as ECM-degradants, anti-inflammatories and ECM building phenotypes occurs. These cells provide the possibility to repair heart tissue through specific biomolecular mechanisms by directly remodelling damaged tissue or by inhibiting or promoting certain properties of fibroblasts.[25] The increase in the number of myofibroblasts as well as the number of capillaries consequently reduces the size of the infarction and increases the thickness of the tissue helping to preserve heart function after MI.[21]

Tissue repair after MI requires an immediate angiogenic response which starts in the border area of the infarction and extends to the nucleus of the necrotic infarction. Thus, after MI, injury, and tissue necrosis lead to the onset of the inflammatory phase, which

consists of intense sterile inflammation, and the dynamic recruitment of various immune cell subtypes, including neutrophils, monocytes/macrophages, dendritic cells, and lymphocytes.[6]

3. Tissue engineering therapies for cardiac repair

After heart damage, repairing cells can be delivered to the heart by implanting an artificial tissue patch or injecting the cell suspension. Direct injection is the most common cell replacement therapy consisting on the injection of cell suspensions using catheters or open chest surgery, and can be applied to different areas of the heart depending on the type of therapy (figure 2).[26]

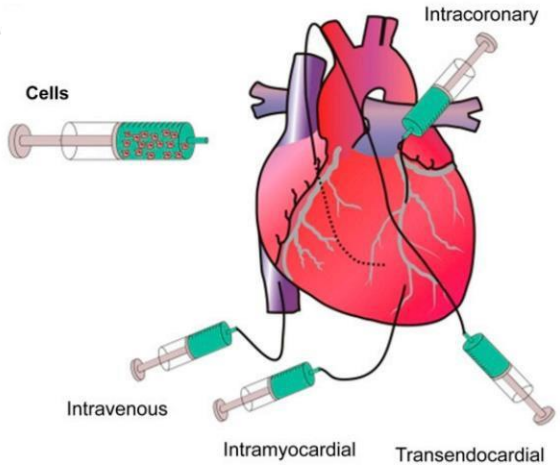

Figure II.2. Various injection locations for cardiac cell injections therapies [Adapted from "Stem Cells Translational Medicine"][26]

TE uses cells as a promising strategy for cardiac tissue regeneration, mainly through the release of paracrine and autocrine important factors that contribute to cell survival, angiogenesis and tissue remodelling.[27] Cell therapies have shown potential for tissue regeneration, but many attempts have failed to show repopulation of damaged tissue due to poor engraftment and survival of transplanted cells. This may be achieved by using injectable materials as cell carriers or seeding cells into scaffolds and patching them onto the heart.[28]

The implantation of tissue patches requires open heart surgery, although patches manufactured with cellular sheet technology can be fixed to the epicardium without using sutures or surgical glues.[29] Thus, cardiac patch is an alternative that solves some of the problems of injecting cells and can also have different applications ways (figure 3).

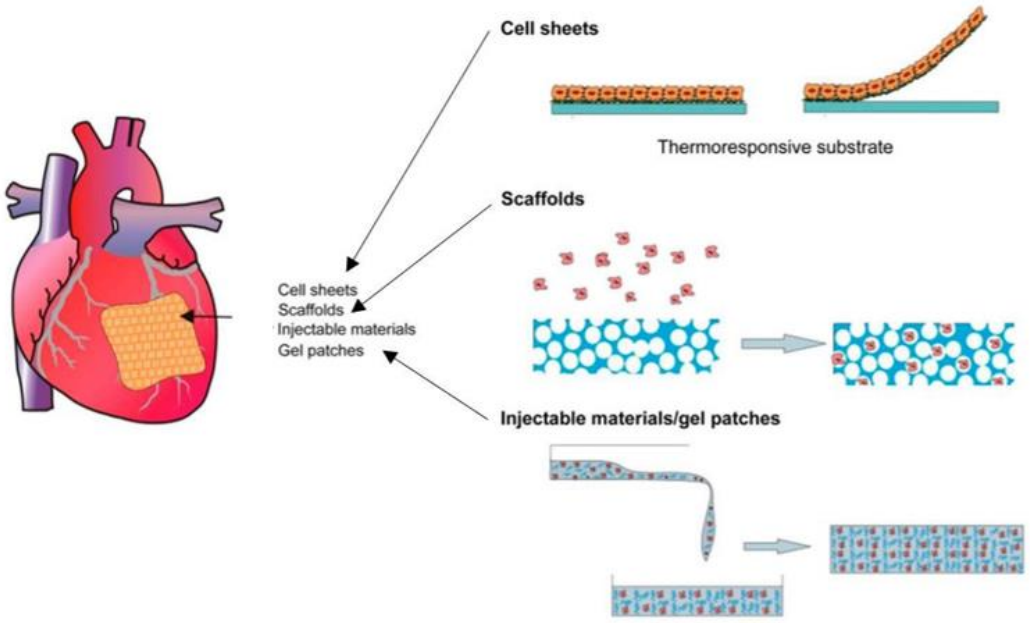

Figure II.3. Various cardiac patch strategies and epicardial patch placement [Adapted from "Stem Cells Translational Medicine"][26]

3.1 Cells for cardiac tissue engineering

3.1.1. Stem Cells

The stem cell is an undifferentiated, self-reproducing cell, which can form specialized cells under controlled conditions. Owing to the generally non-proliferative nature of mature CMs in the myocardium, stem cell-based TE approaches have been extensively explored for cardiac repair. Pluripotent stem cells can give rise to all cells of all tissue types, e.g. embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC).[30]

Different types of adult multipotent stem cells that are proven to be multipotent have been investigated for heart repair. Multipotent stem cells can differentiate in specific types of cells. Most known adult stem cells reside in the bone marrow (BM). These include multipotent HSC, that could regenerate the entire haematopoietic system and MSCs which can differentiate into bone cells, chondrocytes, and fat cells.[31]

3.1.1.1. Human embryonic stem cells (hESCs)

Human embryonic stem cells (hESCs) are derived from the internal cell mass of pre-implantation blastocysts, and they have the potential to differentiate into cell types of all three germinal layers.[32,33] These stem cells can give rise to all the different types of cells in the body and provide an *in vitro* model for early embryonic development, disease modelling and regenerative medicine. Nowadays, there is sufficiently developed technology to be able to establish and expand these cells under animal substance-free conditions, even from single cells biopsied from eight-cell embryos. Notably, some tests confirm genetic stability, absence of tumorigenic mutations, functionality and safety of hESCs.[34]

Studies confirm that three-dimensional (3D) tissue patches made from hESCs-derived CMs (hESC-CMs) have functional properties which can overcome many previous applications for artificial human myocardium and currently provide the closest *in vitro* approximation to native human heart tissue. In sum, the use of non-genetic methods to purify CMs derived from human pluripotent stem cells is a major source of potential for use in cardiac TE.[35]

3.1.1.2. Human induced pluripotent stem cells (hiPSCs)

In 2006, human induced pluripotent stem cells (hiPSCs) were discovered. The discovery that the transient expression of four transcription factors can radically reshape the epigenome, transcriptome and metabolome of differentiated cells and reprogram them into pluripotent stem cells, which has been an important and innovative technological innovation.[36] hiPSCs are biologically like hESCs, and have been an important focus of research. Initially, iPSCs were generated using retroviral transduction. Although effective, this could lead to an oncogenic transformation due to the random insertion of reprogramming reactors. From then on, safer, non-integrating methods such as Sendai virus, mRNA or miRNA, and direct protein delivery, started to be developed.[26] In addition, hiPSC can be generated from patient somatic cells and are immunocompatible for autotransplantation.[37] The iPSC technology allows you to change your cellular destination in the most extreme way possible, i.e., leading to dedifferentiation back to pluripotency. This is a disruptive technology because it is now theoretically possible to produce any type of cell *in vitro.*[36]

The use of hiPSC-derived CMs (hiPSC-CMs) brings new technology to model cardiovascular diseases and advanced regenerative therapies studies. In a research study carried by Florian Weinberger *et al.*, it was possible to create a human-engineered heart tissue functional microtissue from hiPSC-CMs and hiPSC-derived ECs (hiPSC-ECs).[38]

3.1.1.3. Hematopoietic stem cells (HSCs)

Hematopoietic stem cells (HSCs) are an example of multipotent stem cells which can develop into red blood cells, white blood cells and platelets.[31] HSCs renew their population to maintain the potential for long-term blood regeneration, as well as to differentiate themselves from all blood strains. The local BM microenvironment, the "niche", also provides crucial and indispensable factors for self-renewal and differentiation of HSC. In general, this niche controls the quiescence and entry of the stem cell cycle, provides information on the state of the tissues and the body of stem cells, regulates the fate of stem cell daughter cells and reduces the rate of mutation in stem cells.[39]

After MI, HSCs are released from the BM, allowing extramedullary amplification of the myelopoiesis and exacerbating inflammation in the atherosclerotic plaque due to production of externalized splenic and accelerated leukocytes.[40] The development of myoblast sheets to repair the injured myocardium resulted in a reduction of fibrosis through the release of specific GFs to prevent remodelling in association whit recruitment of HSCs.[41]

3.1.1.4. Adult mesenchymal stem cells (MSCs)

Some previous pre-clinical studies using MSCs concluded that they can differentiate into multiple types of heart cells such as CMs, vascular ECs, and smooth vascular myocytes.[42]

For an immediate immune response, MSCs improve cardiac function through the secretion of paracrine factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF).[43] To test the safety and efficacy of cell injection, clinical trials have been conducted with adult and progenitor stem cells, including BM-derived MSCs, peripheral mononuclear cells , and resident heart cells, from which it has been concluded that these cell types are not capable of producing large numbers of CMs. Also, they are thought to secrete paracrine factors such as VEGF, thymosin b4 and stromal-

derived factor, which act directly to induce beneficial effects or indirectly by altering gene expression in the surrounding myocardium.[26]

The presence of heart-specific progenitor cells has led to increased use of these cells. Qiang-Li Wang *et al.* developed a polycaprolactone (PCL)/gelatin cell patch with potential for post-MI therapy by epicardial transplantation.[44] They study the mechanisms of this patch loaded with MSCs on activating endogenous cardiac repair, and 4 weeks after transplantation into murine MI models, the cardiac features were improved, with the migration of engrafted MSCs across the epicardium and into the myocardium.

3.1.2. Cardiomyocytes (CMs)

CMs represent only around 25 to 35 % of the total number of cells in the heart, so they are morphologically and functionally distinct from cardiac non-CM cells which are all cells of the heart excluding CMs. Adult CMs are considered terminally differentiated and proliferate minimally. As such, adult CMs cannot be expanded to enough from cardiac biopsy specimens. All this has led to more research being conducted in this direction by studying several cell types, as previously reported.[26]

After MI, necrotic CMs provide the main stimulus for the inflammatory reaction, releasing damage-associated molecular patterns (DAMPs) into the infarcted area. Surviving CMs in the borderline infarction area can also trigger an inflammatory response by producing and secreting cytokines in response to activation with interleukin-1(IL-1), TLR ligands, or ROS. Some immunohistochemical studies and *in situ* hybridization experiments have led to the conclusion that viable CMs in the borderline infarction area express the intercellular adhesion molecule and can synthesize cytokines and chemokines.[20] Cell sheet engineering is a useful technology to produce myocardial tissues for clinical application to promote regeneration. Layered CMs sheets already prove to be a great potential for post-MI injured areas repair scaffolds due to long survival, contractile properties, and structural properties similar to the native heart tissue.[45]

3.1.3. Endothelial Cells (ECs)

ECs form a crucial link between the cardiovascular system and the immune system and are therefore a constituent part of the heart and vasculature. ECs not only participate in angiogenesis, hemostasis and vascular tone regulation but are also an essential and active component of immune responses.[46]

In the heart of adult mammals, ECs are the most abundant non-CMs. Understand CM-ECs interactions is critical to understand cardiac function and ultimate cardiac repair. Several studies have demonstrated that angiogenic stimulation supported by ECs can promote CM survival.[47] Recently, Maiullari *et al.* engineered a vascularized heart microtissue by encapsulating ECs and CMs in an alginate based hydrogel.[48] The results suggested that the pre-formed vasculature has the potential to improve the integration of the engineered cardiac tissue with host's vasculature.

3.1.4. Fibroblasts

Cardiac fibroblasts are important for development, depositing collagen and other ECMs components and they are defined as cells that produce connective tissue. Unlike connective tissue of bone and tendon, which is organized into regular collagen patterns, cardiac ECM is dense, irregular, and composed of collagens, proteoglycans and glycoproteins.[49] In adult hearts, the fibroblasts are constantly modifying the microenvironment, degrading and depositing ECM. Fibroblasts are also responsible for cardiac fibrosis, which is the accumulation of ECM in response to a pathological stimulus. When the fibroblasts are activated by lesions or inflammation, collagen production begins to be regulated to stabilise the heart. While ECM deposition can initially strengthen the integrity of tissue, extensive fibrosis can impair heart function. Persistent fibrosis can ultimately lead to HF.[50]

During the process of cardiac repair, cardiac fibroblasts are very important due to their proliferation which is usually accompanied by the recruitment of blood vascular ECs. Although this process is a potential therapeutic target for improving cardiac neovascularization having a wide impact on the field of vascular biology and cardiac regeneration, previous studies using other genetic tools have shown that resident

fibroblast strains mainly measure cardiac fibrosis, suggesting that most fibroblasts adopt immediately their destination after a cardiac lesion.[51]

Robert S Kellar *et al.* used human fibroblasts to create a 3D scaffold as a cardiac patch to stimulate revascularization and improve function of the infarcted LV in a mice model.[52] This patch showed a functional and viable to stimulate a regenerative response within an area of infarcted cardiac tissue due to potential angiogenic GFs presents in cells.

3.2. Biomaterials for cardiac tissue engineering

To mimic the native cardiac environment, a biomaterial must take into account several characteristics such as biocompatibility, biodegradability, elasticity and rigidity for adequate biomechanics, electrical properties that resist strong muscle contractions, a necessary structural that allows both adhesion and cell proliferation and helps in remodelling and alignment of CMs.[53] The biomaterials used for TE applications can be categorized in two main groups: synthetic and natural derived biomaterials.

3.2.1. Synthetic sources

Synthetic materials such as biodegradable polymers, despite their initial inert and non-bioactive properties, have been used in TE due to the potential to produce surfaces with controlled structure and no immunological problems. However, chemical modifications are required to functionalise them to allow the best possible mimicry of the diversity and complexity of the native tissue ECM.[54–56] Below are described some of the main polymers from synthetic origin that have been used in cardiac TE applications.

3.2.1.1. Poly(ethylene glycol) (PEG) is a hydrophilic and biocompatible polymer widely used in TE research to form biomaterials, such as hydrogels due to its chemical structure constituted by a diol and two hydroxyl end groups, which can be converted into other functional groups. Thus, this synthetic material, highly inert, is usually functionalized with bioactive domains.[57] Pre-designed patterns on PEG were used in a study by Gozde Basara *et al.* to create an electrically conductive 3D printed composite construct for cardiac TE.[58] This study shows great potential in the use of this platform to create physiologically

relevant cardiac patches for the treatment of MI, due to conductive and topographical features which allow a close mimic of the ordered structure of the native ECM and the electroconductivity of the human heart.

Poly(ethylene glycol) diacrylate (PEGDA) is a type of polymer derived from PEG that can be applied in the manufacture of hydrogels via radical polymerization of acrylate terminals.[59] By using light exposure and a biocompatible photoinitiator PEGDA is quickly crosslinked. James Moon *et al*. carried out a study with ECs in which the objective was to regulate and guide angiogenesis in PEGDA hydrogels.[60] Techniques such as the two-photon laser scanning lithography allow the formation of micropatterns in 3D structures for cell culture studies. Studies prove that PEGDA hydrogels, due to their adjustable mechanical properties, allow them to be patterned to produce anisotropic structures for studies of cellular response to the substrate.[60]

3.2.1.2. Poly(lactic-co-glycolic) acid (PLGA) has long been used to manufacture devices for drug administration and TE applications as it is a biocompatible and biodegradable polymer with adjustable mechanical properties.[61] In the study of Yin Chen *et al.* a thin film of PLGA was successfully fabricated with microgrooves that can be used in the construction of a biomimetic cardiac patch.[62] This cardiac patch shows how the use of this micropattern manages to mimic the anisotropic electrophysiological characteristic of native cardiac tissue much better than its absence.

3.2.1.3. Polylactic acid (PLA) despite being limited by hydrophobicity or by having a slow degradation rate, has been increasingly used for biomedical application due to its properties such as biocompatibility, biodegradability, and mechanical resistance. This biomaterial can be combined with other polymers and allow the development of materials with the desired properties for a specific application.[63] PLA was reported, for the production of nanofibrous scaffolds in composites with chitosan and silk fibroin.[64,65] These studies conclude that the developed nanofibrous bioactive scaffolds have good mechanical properties and swelling activity that make these biomaterials biocompatible and efficient for effective implantation in the heart.

3.2.1.4 Polycaprolactone (PCL) is a highly biocompatible and biodegradable polymer, being also widely available, cost effective and easy for processing and chemical modification. In a study using scaffolds incorporating PCL with double-layer hydrogels, it

was possible to demonstrate that stem cell encapsulation allowed the release of anti-inflammatory cytokines for the reconstruction of damaged cardiovascular tissue.[66] Seokwon Pok *et al.* have achieved interesting results showing how a multilayer myocardial patch made of a PCL membrane supporting a chitosan/heart matrix hydrogel promotes muscle and vascular remodelling.[67]

3.2.1.5 Polyglycerol sebacate (PGS) has been widely used in TE due to its biocompatibility and elastomeric properties, thus being an interesting polymer to be used in cardiac TE. George C. Engelmayr Jr *et al.* reported the use of microfabrication techniques to create an accordion-like honeycomb microstructure with controllable stiffness and anisotropy similar to the native myocardium tissue.[68] The results demonstrate that these structures promote the formation of interconnected neonatal rat heart cells into the scaffolds pores and elongation through the surface constituting a biomimetic microstructure with cell alignment and mechanical properties closer to the existing in rat cardiac tissue.

3.2.1.6. Polyurethane (PU) is the most popular polymer used for biomaterials to clinical applications, being usually used as biostable and inert material in heart valves, vascular grafts, catheters, and prostheses. These tuneable mechanical, physical and biological properties such as biodegradability and biocompatibility, make these materials desirable in TE, showing ability to promote cell adhesion and proliferation without adverse effects.[69] Biodegradability in PUs can be achieved by chemical synthesis through the incorporation of hydrolyzable segments into their backbones.[70] This synthetic material was used for example by Alperin *et al.* in a study consisting of the culture of ESC-derived CMs (ESC-CMs) on thin PU films to be coated with gelatin, laminin or collagen IV in order to enhance cell adhesion. Results show that surfaces have good mechanical properties to support cell culture and contractile properties are developed in myocytes, making them platforms with potential for in vitro cardiac-mimicking tissue.[71]

3.2.2. Natural sources

Natural biomaterials are used in TE due to its biocompatibility and bioactivity allowing studies to cell adhesion, migration, proliferation and differentiation and the clinical application to replace or restore structure and function of injured tissues.[72–74]

3.2.2.1. Alginate is a natural hydrophilic and biodegradable polymer that, as it mimics the extracellular matrix and supports cellular and metabolic functions, became one of the first biomaterials tested in pre-clinical research and clinical trials in cardiovascular diseases.[75,76] In a research carried out by QuanQi *et al.* a therapeutic strategy is proposed, consisting at a local intramyocardial delivery of a bioglass with alginate hydrogels with beneficial properties in angiogenesis, presenting itself as a potential biomaterial in post-MI cardiac regeneration.[77] Alginate-derived biomaterials are already being used in clinical trials for cardiac regeneration. It is the example of a study carried out by Natali Landa *et al.* which was show, for the first time, an injection of in situ-forming scaffold constituted by a bioabsorbable alginate hydrogel.[78] This implant demonstrates the attenuation ability of adverse cardiac remodelling and dysfunction in the post-MI cardiac infarcted tissue of rats. Studies have not only been carried out in animals but also in humans with some medical devices developed. Alginate clinical approaches in the LV wall showed reduction of stress of the dilated heart wall improving mechanical properties and having favourable biological effects in tissue regeneration and neovascularization.[79] Alginate-derived hydrogels can be used for implants, and results shows how these biomaterials injections are more effective compared to standard medical therapies for patients with advanced chronic HF. This is demonstrated, for example, with the Algisyl-LVR ™ implantable hydrogel, which in combination with coronary artery graft surgery (CABG) showed changes in myofiber stress at end-diastole and end-systole, making it more uniform in the LV.[80,81]

3.2.2.2. Hyaluronic acid (HA) is a linear and non-immunogenic polysaccharide that can be found in the ECM of different tissues, being involved in regenerative processes and cell signalling. Biological and structural properties of HA make it a very important key in the construction of new biomaterials for use in myocardial regeneration, which have shown good results in the induction of neovascularization.[82,83] Jeong Yoon *et al.* evaluate the efficiency of an injectable HA-based hydrogel in post-MI functionality recovery of the heart.[84] That was considered a promissory treatment due to its easy-to-manufacture and applicability by simple injection into the epicardium with great regenerative results.

3.2.2.3. Collagen is another natural material that has already shown good results in scaffold-based strategies for cardiac tissue regeneration. Collagen is one of the most abundant proteins in the ECM of heart, playing a very important role in the structural and

functional organization of myocardial tissue. These characteristics, together with a good mechanical dehydration of a collagen hydrogel, for example, make it possible to mimic the stiffness environment suitable for cell adhesion and proliferation, allowing a greater similarity with the host tissue, as well as its contractile network formed by the CMs.[85,86] A study by Yang Liu *et al.* combined high concentrations of elastin and collagen with PCL to produce hybrid electrospun nanofibrous sheets.[87] The good cytocompatibility to deliver transplanted stem cells to injured areas post-MI and the good mechanical properties make this approach a potential cardiac-mimicking patch.

3.2.2.4 Gelatin it has very good biocompatibility and is easy to process, still it has a poor mechanical performance and a fast degradability, which leads to its being combined with other materials. Seokwon Pok *et al.* presented a multi-layer hydrogel scaffold that combines gelatin and chitosan supported by a PCL core.[88] This combination makes this biomaterial with mechanical strength sufficiently suitable for application as a cardiac adhesive. In other research, carried out by Soah Lee *et al.* highlight how the chemical crosslinking of gelatin makes possible the formation of a microenvironment that allows cell-cell communication and suitable mechanical properties for the engineering of human cardiac microtissues through the encapsulation of CMs derived from hiPSC-CMs.[89]

3.2.2.5 Fibrinogen is a soluble macromolecule that, after a chain of reactions that culminates in the action of the serine protease thrombin, is converted into fibrin, which is an insoluble fibrous protein with an important role in blood coagulation, in the inflammatory response and in wound healing.[90] Fibrin patches with iPSC-derived CM spheroids were implanted in rats after MI. Results have shown that the fibrin matrix with a spheroid encapsulation had a higher engraftment rate than direct intramyocardial injection (more than ~ 25% vs. less than ~ 10%, respectively).[91] The biophysical properties of fibrin, such as porosity, stiffness, and biodegradability made this material interesting for use as a biopolymer to support cardiac TE. Studies show that the combination with other materials such as collagen or gelatin, for example, allows to obtain platforms with improved mechanical properties and that demonstrate an optimized cellular activity in biological assays.[92,93]

3.2.2.6. Matrigel is a solubilized basal lamina protein-enriched extract isolated from Engelbreth-Holm Swarm (EHS) mouse sarcoma, constituted mainly by laminin and collagen IV.[94] This material is very promising in cardiac regeneration field due to its structural similarity to the extracellular matrix and for promoting the angiogenesis of myocardial tissue after an injury. The efficacy of intracardiac injection of matrigel was tested by Lailiang Ou *et al.* and showed a good induction of cell recruitment as well as an improvement in the functional properties of the tissue.[95]

3.2.2.7. Decellularized extracellular matrix (dECM) is an effective biomaterial in the repair of cardiovascular tissue as it has proteins, glycosaminoglycans, proteoglycans and many other components that belong to the native tissue matrix, making its regenerative capacity of enormous interest with many possibilities of biomimicking-tissue fabrication.[96–98] These properties have already been investigated with seeding of CMs derived from ESCs and from iPSCs into dECM, with results showing that it is a suitable material for the formation of suitable structural and functional platforms for application in cardiac engineering.[99,100] Cardiac patches combing dCECM and gelatin methacrylate (GelMA) demonstrated that encapsulated cardiac progenitor cells remain viable and showed increased cardiogenic gene expression, compared to those grown in pure GelMA patches. *In vivo* studies demonstrated that patches integrate with the native myocardium.[101]

4. Micropatterned biomaterial surfaces to control cell fate

Cell fate can be influenced by both biochemical and biophysical factors. Thus, the surrounding microenvironment and factors such as direct communications between cells and neighbouring cells, the composition of the ECM, stiffness, topography and mechanical stimulation are widely investigated for their influence on cellular behaviour.[102–106] In this cellular process, the biophysical properties of the microenvironment are converted into biochemical signals, involving several pathways through integrins, focal adhesions and actin cytoskeleton that alter cell behaviour.[107–109] This natural cells' behaviour, has inspired many research in the biomedical field to design and create biomaterials with specific topographies to control cell function.[110]

Native myocardial tissue has mechanical and electrical properties derived from anisotropy resulting from the orientation of muscle fibers.[111] In particular, cardiac TE

critically depends on the ability to create appropriate structural organisation of CMs, fibroblasts, and EC, the major cell populations found in the native cardiac tissue. Researchers in the field cardiac TE have developed a particular interest in designing platforms with specific topographies that will orientate cell organization and function.[112] The microfabrication of patterns on substrates where cells can be cultivated allows to obtain scaffolds with biomimetic microenvironments with applications such as tissue regeneration and stem cell differentiation.[113,114] Surface topographical cues can be used to align cells and create the organized hierarchy of cardiac native tissue that is highly aligned in aforementioned anisotropic laminae.[115,116] In the following sections are presented and discussed some of the main techniques that have been explored to design topographic surfaces that have been used to direct cell function in cardiac TE.

4.1. Micropatterning techniques

Micropattern fabrication technologies derive from the semiconductor industry, enabling the creation of patterns with high resolution and fidelity at scales ranging from submicron to a few centimeters.[117–119] In TE these technologies have been applied for their reproducibility and good results in the consistency of patterns created on substrates that allow cells to proliferate and guide themselves according to the applied topography, being promising platforms in the area of cardiac regeneration where alignment is a crucial factor for cellular interactions and the creation of a microtissue.[116,118]

4.1.1. Photolithography

Photolithography is a patterning method which allows the transfer of geometric patterns from a mask to a substrate through ultraviolet (UV) light exposure. Basically, a photoresist is placed on the surface of the substrate which, when receiving UV light through a mask that contains the desired patterns, forms a pattern on the substrate itself that can be used as a mask or master for any biological materials.[120] In the application of this method the resolution is limited by the diffraction limits of the light source, and for example nano-scale patterning using photolithography requires a short wavelength light source combined with sophisticated optics. Thus it is a technique that can resort to greater complexity and cost when it is adapted for certain kind of patterning.[121]

Using photolithography, it is possible to create patterns that guide cell interactions, and the way cells grow and obtain an anisotropic surface similar to the ECM of cardiac tissue allowing the culture of CMs and their organization and alignment along the pattern.[122]

4.1.2. Soft Photolithography

The soft lithography technique is a low cost, easy and accessible technique to fabricate biomimetic patches. In this method a soft polymeric stamp is used to transfer micro, or even nano, scale patterns to surfaces allowing different moulds to micropattern different structures.[123,124]

The polymeric stamp is typically made of (PDMS) that is biocompatibility and has suitable thermal, mechanical, and optical properties. Thus, this technique is widely used in scientific research and the mold can be reused for several tests, making it a cheaper alternative that allows a great resolution through an easier method.[125]

In a study carried out by Julia Dahlmann *et al.*, soft lithography was used to prepare agarose surfaces with microwells for the formation of aggregates of murine-iPSCs and hESCs, which showed good differentiation in the cardiac lineage.[126] This study demonstrates the effectiveness of this method in the formation of micropatterns that allow the change of cell behaviour in a controlled way, being a great advantage for cardiac TE.

The alignment and orientation of cells on a scaffold where they are cultured are affected by the topography, and by varying the pattern structure type, the contact guidance will have a different influence on how cells proliferate. The alignment of CMs is important in native tissue to maintain the functional and mechanoelectrical functions that generate cardiac contractions. Raghavendra Palankar *et al.* carried out a study to understand the influence of micropillars in the mechanobiology of hiPSCs-CMs (figure 4).[127]

This research shows how micropillars induce the reorganization of the cells' structure, allows the understanding of cardiac cell interactions with a microenvironment with mechanical support to contact guidance. These interactions are an important achieve to fabricate a scaffold for guiding cells in the native injured tissue.

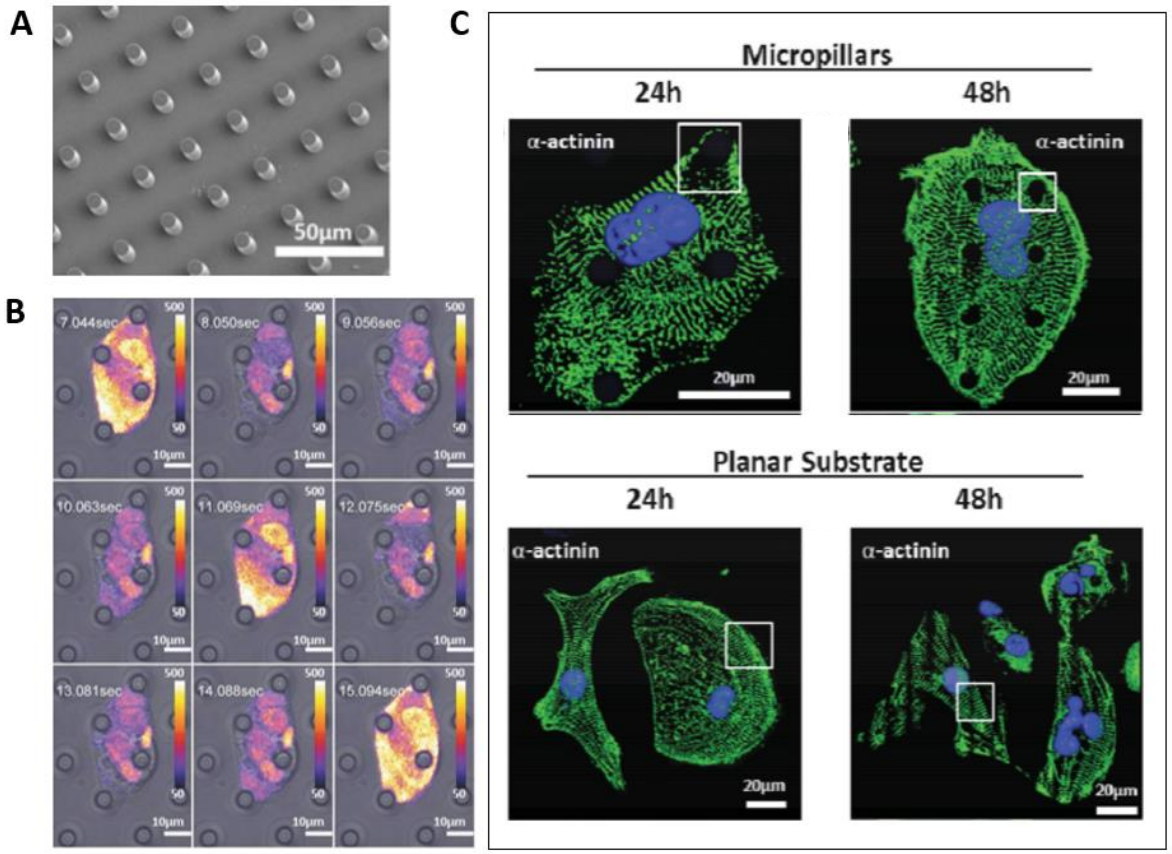

Figure II.4. SEM image of 3D micropillars (A),confocal laser scanning microscopy images where the pseudocolored fluorescence intensity scale indicates the changes in cytosolic calcium during cardiomyocyte contraction (B), and confocal immunofluorescence images of subcellular arrangement of α-actinin in iPSC-CM cultured on micropillars and planar substrates (C). Adapted from [127].

Other study has used micropillar patterns (figure 5A) to produce a structure which allows cells culture and their organization and elongation analysis (figure 5B). MSCs and CMs were confined in this biomimetic microenvironment shown elongation and alignment along the stiffer axes of micropillars with different dimensions and arrangements, with better directionality in anisotropic feature.[128] That type of CMs organization provides to this scaffold the potential to have a highly organized and functional cardiac tissue.

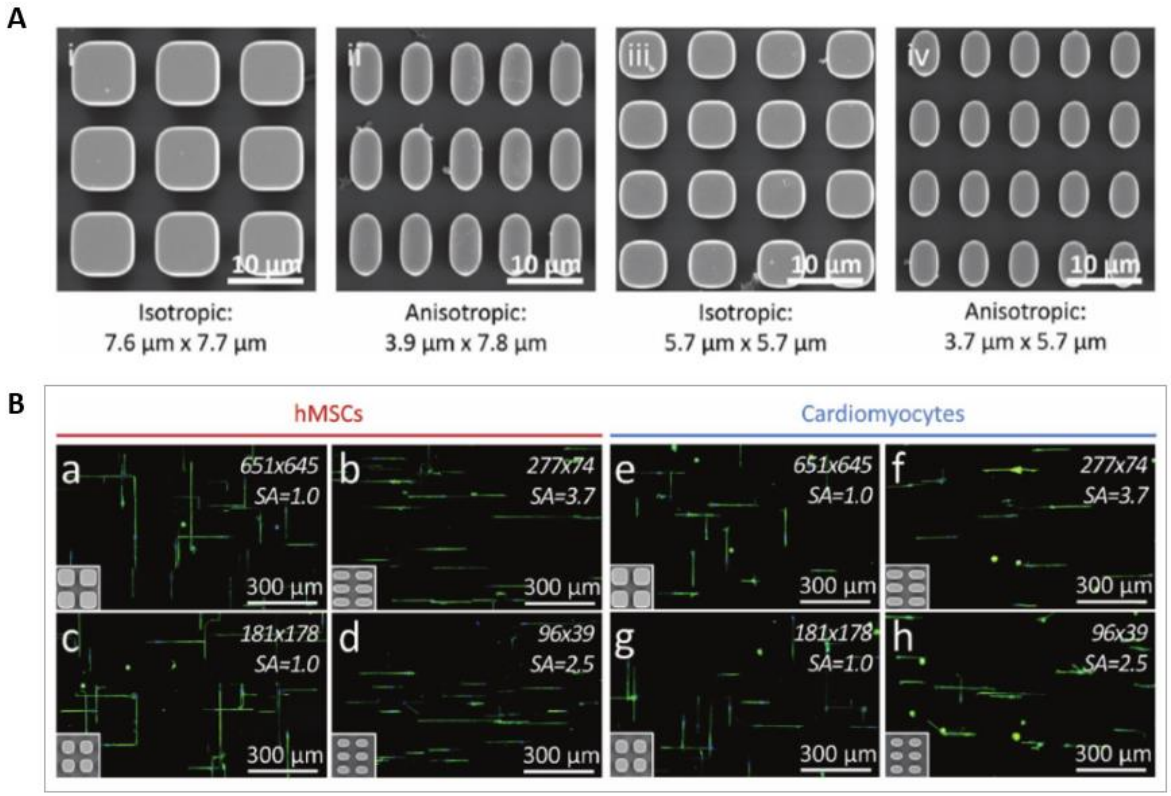

Figure II.5. Micropillar arrays fabricated with different isotropic an anisotropic topography dimension (A) and representative images of MSCs and CMs alignment between them labelled by cytoskeletal F-actin and nuclear staining (B) Adapted from [128].

4.1.2.1. Replica Molding

Replica molding is a technique that allows to duplicate the pattern of three-dimensional structures in a single step. A PDMS mold with the topography of interest is used to transfer the pattern to the surface of another polymer. The prepolymer is deposited in the PDMS mold by casting or coating by rotation, which consists of rotating the substrate at high speed, spreading the material uniformly over the intended surface. After curing, the prepolymer is separated from the mold by peeling.[129,130]

Ewelina Tomecka *et al.* proposed a microsystem that allows 3D and 2D human cardiac cell cultures for 4 days.[131] Results showed a better proliferation of cardiomyocytes proliferated in 3D than 2D conditions. The cytotoxic effect of isoproterenol was also examined on cells cultured in the microsystem demonstrating to be a promising microfluid cardiac model for drug analysis. The presented microsystem consists of two inlets and two outlets connected with a microchannel structure in PDMS fabricated by replica molding using a poly(methyl methacrylate) (PMMA) master.

4.1.2.2. Microcontact Printing

Microcontact printing is the only soft lithographic technique capable of generating chemical patterns on a surface with detail at the molecular level. For the fabrication of micropatterned surfaces, microcontact printing uses topographically patterned PDMS stamps which is placed above the substrate, and due to the elasticity of the stamps the patterning can happens at non-planar surfaces, such as porous, rough, or curved ones.[129]

Max R Salick *et al.* used high-resolution photolithography techniques and microcontact printing to produce 2D micropatterned features with hESC-CMs seeded in a controlled way.[132] The highly-aligned cell aggregates showed great potential to be part of cell-based pharmacological studies and to help the understanding of ECM geometries influence in myofilament structure and maturation in hESC-CMs.

Through sequential preculture of ECs, fibroblasts and CMs, Rohin K Iyer *et al.* develop capillary-like cords in Matrigel-coated microchannels.[133] Modulating the percentage of ECs, this study shown results which highlight the potential of vascularization of these cells, and the importance of this property to achieve an engineering tissue with similar electrophysiological properties to native myocardium. Micropatterned metacrylated tropoelastin hydrogel patches containing aligned microchannels, made through molds fabricate by a microconntact printing technique, mimicking those of the native myocardium, have shown to promote aligned attachment, spreading, function and intercellular communication of CMs, oppositely to the unpatterned hydrogel in which CMs randomly attached and the cardiac function early decreased (see figure 6).[134]

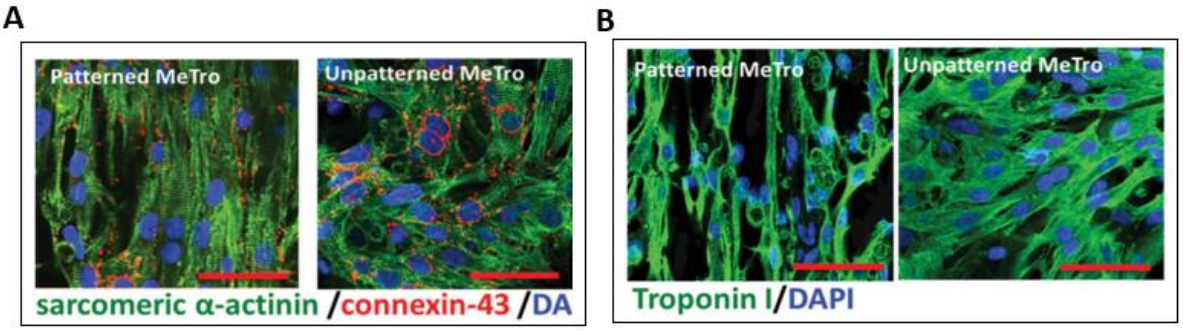

Figure II.6. Troponin (green)/nuclei (blue) (A) and sarcomeric α -actinin (green)/ connexin-43 (red)/nuclei (blue) (B) stainning on day 8 of culture of CMs in patterned and unpatterned metacrylated tropoelastin (MeTro) gels Scale bar = 50 μm. Adapted from [134]

4.1.2.3. Microfluidic patterning

Microfluidic patterning allows to design and creates biologically suitable cellular arrangements being an interesting technique for tissue biomimetics construct tissues.[135] Microfluidic patterning consists of the use of PDMS mold with channels embossed that serves as templates to the pattern. This PDMS layer is placed in contact with a glass or a polymer surface forming the limit layer of the channel. Micropattern is formed through the restriction of fluid flow, deposited in the substrate, to the desired areas. The fluid can't be put under pressure due to the weak physical seal between the two layers mentioned what can make the liquid escape from the channels.[129]

Microfluidic patterning was used for example by Ali Khademhosseini *et al.* to fabricate patterned HA substrates to induce the generation of 3D cardiac organoids assembly.[136] Firstly, cardiomyocytes showed elongation and alignment along the pattern's direction, and after 3 days of culture, the cells detached and formed contractile cardiac organoids, creating a successful in vitro cardiac tissue model.

This technique was also adapted for a study by Nasim Annabi *et al.* where a PDMS-based microfluidic coating method with methacrylated tropoelastin (MeTro) and GelMA was developed to study the adhesion and orientation of CMs within microchannels. It was verified elongation of the CMs along the generated microchannels, creating aligned cardiac fibers, demonstrating itself as a potential platform for cardiac TE.[137]

Microgrooves has proven to be an appropriate geometry to be patterned in matrices to ECs culture, provide a system which enable the formation of microvessel-like structures.[138] Ali Navaei *et al.* proposed GelMA hydrogel with a microgrooved topographical feature (see figure 7).[139] This study demonstrates to have a potential functional cardiac patch to injured tissue clinical application post-MI, due to the additional incorporation of electrically conductive gold nanorods (GNRs) that provides a better mimicking of the native myocardium tissue electrical functionality.

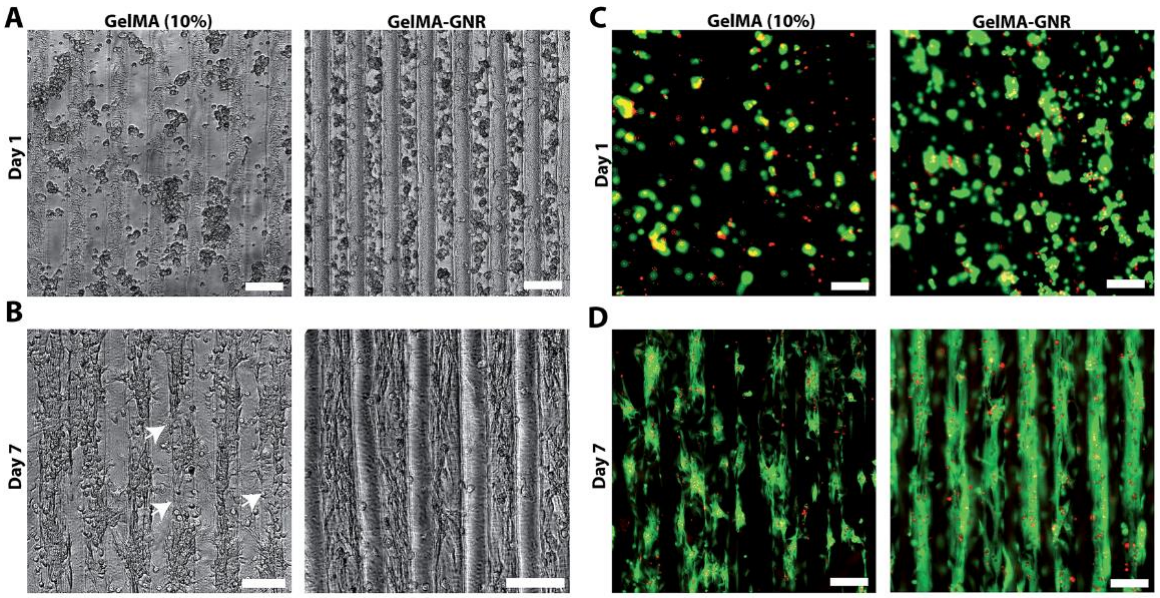

Figure II.7. Phase-contrast images of cardiac cells seeded on GelMA and GelMA–GNR microgrooved hydrogel on (A) day 1 (B) and day 7 of culture. White arrows represent the disconnect between cellular clusters. Fluorescent viability images of GelMA and GelMA–GNR microgrooved cardiac tissues on (C) day 1 and (D) day 7. Live cells are stained in green and dead cells are stained in red. Scale bars:100mm. Adapted from [139]

Conclusions and future directions

Despite all the highly orchestrated events that follow a myocardial injury, the heart has a very limited capacity to self-repair. Thus, the use of TE to treat cardiovascular damage has emerged as a research topic in the past few decades. This implies the combination of cells, signalling molecules and biomaterials to engineer a functional tissue that can exert beneficial effects on heart function after implantation. Natural and synthetic based materials have been explored in cardiac TE, however, the ideal substrate with all essential requirements does not exist. The surface topography of a biomaterial-based scaffold can modulate cell adhesion and function. Several microfabrication techniques have been developed in recent years with the main objective of obtaining complex platforms with specific topographical cues to direct cell culture and function in multiple applications. Microtopography has shown good results in fabrication of biomaterials influencing the cell contact guidance, allowing better alignment and organization of the tissue formed as well as communications between cells. These properties are important key factors to create a microenvironment the better similar to the anisotropy existing in native cardiac tissue and subsequently to potential future clinical application in cardiac regeneration.

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Chapter III

Materials and Methods

Materials and Methods

1. Synthesis of Methacryloyl Platelet Lysates

Methacryloyl platelet lysates (PLMA) are formed by reaction of methacrylic anhydride 94% (Sigma-Aldrich, Germany) with amino and hydroxyl groups of proteins present in human platelet lysates (PLs).[1]

PLs are classified as a raw material of biological origin which is an efficient xeno-free alternative to growth medium supplements like fetal bovine serum (FBS) for both isolation and propagation of human cells.[2] It is a mix of growth factors and bioactive proteins involved in cell proliferation and maintenance. [3] PLs are obtained from a blood-derived concentrate so-called platelet-rich plasma (PRP) by freeze-thawing and centrifugation steps which results in the lysis of platelets and consequently release of their content (PLs).[4]

PLs (STEMCELL Technologies, Canada) were thawed at 37 °C and to 10 mL (total protein content 6.3 g dL^{-1}) of this solution, 100 µL of methacrylic anhydride were added in order to synthesize PLMA. The reaction was carried out under constant stirring at room temperature. The synthesized PLMA was then purified by dialysis against deionised water for 24h to remove the excess of methacrylic anhydride. The PLMA solution was filtered with a 0.2µm filter to sterilize it, frozen with liquid nitrogen, freeze-dried, and stored at 4°C until further use (Figure 1).[1]

Due to the presence of methacryloyl groups in PLs, it is possible to fabricate PL-based hydrogels just by exposure of a PLMA solution to UV light. Moreover, such modification allow the fabrication of hydrogels with increased mechanical properties and great biological features.[1]

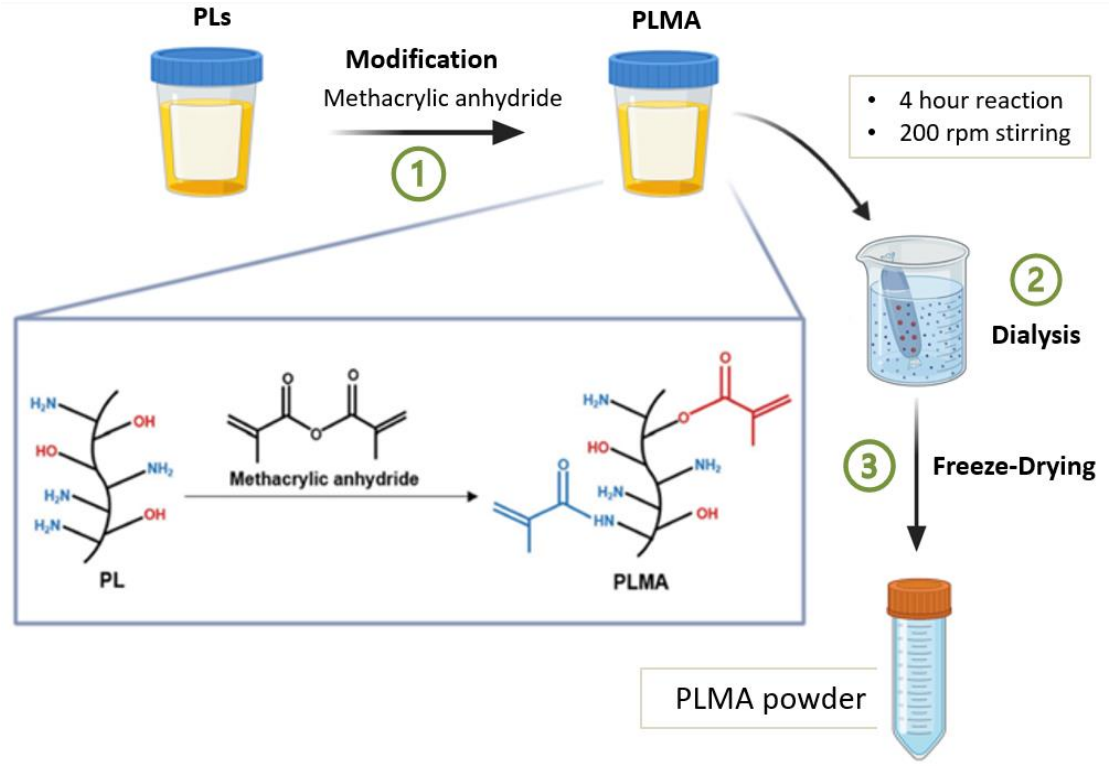

Figure III.1. Schematic representation of PLMA synthesis by (1) modification of PLs with methacrylic anhydride followed by (2) dialysis to remove the excess of methacrylic anhydride and freeze-drying (3).

2. Preparation of PLMA hydrogels with different topographies

Polydimethylsiloxane (PDMS) is a silicon rubber that is commonly used in biomaterials engineering to fabricate micro-devices that has already been studied to understand cell behaviour in controlled microenvironments with unique mechanical properties and specific topographies.[5–8] PDMS is prepared by mixing the curing agent in a base polymer (Sylgard™ 184 Silicone Elastomer Kit, Dow Corning) following a 1:10 ratio. These two components are mixed and the final mixture is degassed in vacuum for 30 minutes to remove any remaining air bubbles and placed in an oven at 60ºC for 2h for curing.[9,10]

In this work, PDMS was used to produce molds with different topographies to be used as counter molds for hydrogels fabrication. Two different PDMS micropatterned patches with microwells were prepared by a double cast moulding process according to a protocol

previously reported[11]: one with an hexagonal arrangement and another with a square arrangement– see figure 2A.

For both arrangements, the microwells have a depth of 100 µm, diameter of 60 µm but different spacing between them: 40 µm and 80 µm.

As previously said, such PDMS molds were produced to be used as counter molds to form micropatterned hydrogels. In this sense, hydrogels derived from PLMA with different topographies will be produced. To do so, lyophilized PLMA was dissolved in a solution of 0.5% w/v Irgacure 2959 (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS, Thermo Fischer Scientific, USA) to final concentrations of 10%, 15%, or 20% w/v of PLMA. Afterwards, PLMA hydrogels were made by pipetting the final solution to a PDMS mold and the micropatterned patch previously mentioned was placed above the PLMA solution – see figure 2B - followed by UV exposure (0.95 W cm^{-2}) for 10 min. When the PDMS micropatterned patch is placed on top of the PLMA solution to serve as counter mold it is expected that this solution will run along the microwells and after photocrosslinking the PLMA hydrogels will acquire the micropatterning with micropillars. To help PDMS patch detachment from PLMA hydrogels and to maintain the micropillar structure, the PDMS micropatterned patches were washed with ethanol, dried, and finally taken to a hydrophilic treatment in a ATTO low-pressure plasma system (Diener) for 7 min (70V, 0.4-0.6 mbar). During plasma treatment, a high energy medium under low pressure air, makes all neutral particles unstable and their division into negative and positive charges or electrons excited on the left. This technology is used in PDMS to turn the surface into a hydrophilic state due to the SiO_2 layer formed. The layer formed on the surface disappears after some time and a new treatment is necessary to eliminate the natural hydrophobic properties of this material.[12,13]After detachment of the PDMS micropatterned mold, PLMA hydrogels with micropillar are obtained. These hydrogels have a square shape with an area of 1 cm^2, but afterwards four circles with a diameter of 6mm are cut with a biopsy punch (Kai Medical, Japan).

This methodology is described as soft photolithography that allows a low cost and rapid fabrication of micropatterned platforms, with production of structures from nano to micro-scale, such as biomimetic patches. Briefly, it is used a stamp, made for example from PDMS, to transfer micro, or even nano-scale patterns to surfaces, allowing the production of different topographies.[14–16]

Soft photolithography is a simple procedure that can be applied to several materials, allowing its use in biochemistry and biology, and it does not require a very strict control over the environment in which they are manufactured, beyond what is necessary for cell culture.[17] The use of a PDMS mold in this technique allows you to take advantage of its flexibility, which makes its detachment from the substrate more easily and efficiently, without damaging the microstructure formed by UV crosslinking.[18]

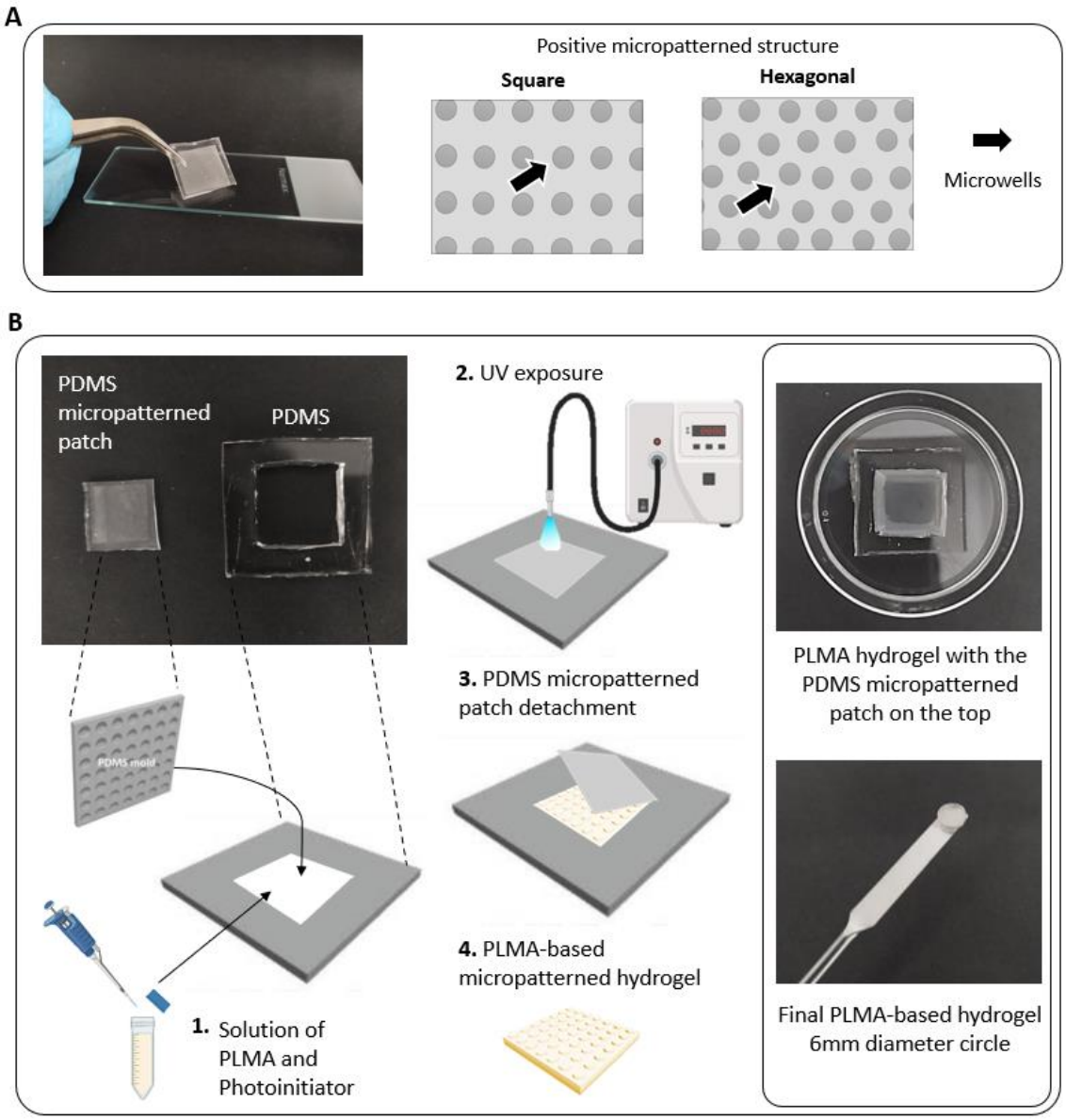

Figure III.2. (A) Schematic illustration of microstructure patch with hexagonal and square geometrical arrangements of the microwells and (B) PLMA-based micropatterned hydrogels formation by a soft photolithography process using the PDMS micropatterned patches. Some illustrative images adapted from [19]

3. Mechanical properties of PLMA micropatterned hydrogels

Mechanical properties of hydrogels are one of the key features that must be assessed during their development as they will influence cell behaviour. Depending on the used polymer and crosslinking density, hydrogel mechanical properties should be adjusted to prepare hydrogels with the desired features able to promote cell maintenance and new tissue formation. Moreover, by tuning the mechanical properties of the hydrogels, it is possible to closely mimic native tissue stiffnesses.[20–22]

Mechanical assays were performed based on compression tests employing an Instron Universal Mechanical Testing Machine 3343 (Instron, USA) equipped with a load cell of 50 N. The cylindrical hydrogels specimens, prepared the day before and placed in PBS overnight, were tested at room temperature. For each hydrogel, the displacement data from initial until rupture load were measured using a computer data acquisition system connected to the tester (Bluehill® Universal, Instron). The Young's modulus was defined as the slope of the linear region of the strain–stress curve, corresponding to 0–5% strain. Ultimate stress and ultimate strain values were taken as the point where the failure of the hydrogel occurred.

4. In vitro cell culture of cardiomyoblasts and endothelial cells

In vitro cell culture assays were performed in order to validate the fabricated micropatterned PLMA hydrogels for cell culture, in particular, for culture of cardiomyoblasts and endothelial cells.

Cell culture assays on micropatterned PLMA hydrogels were performed with human umbilical vein endothelial cells (HUVECs, ATCC) and myoblasts derived from embryonic myocardium rat tissue (H9c2(2-1), ATCC). HUVECs were cultured in Medium 199 (Sigma-Aldrich) supplemented with 20% FBS, 1% antibiotic/antimicotic, 1% of glutamate, 1% v/v of Heparin (100 mg/mL, Sigma-Aldrich), and 0.1% v/v of Endothelial Cell Growth Supplement (40 mg/mL, Merck, Germany). HUVECs were used until passage 8. Cell suspensions were prepared by trypsinization with a trypsin/EDTA solution (Sigma-Aldrich, Germany). H9c2(2-1) were cultured in Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG, Sigma-Aldrich) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic.

Cells were cultured under 5% CO_2 atmosphere at 37 °C (standard culture conditions) fed every 2–3 days, and sub-cultured when reaching 70–80% confluence as to prevent the loss of adherence as differentiation potential. Cell suspensions were prepared by trypsinization with a TrypLE™ Express solution (Thermo Fisher Scientific).

Micropatterned PLMA hydrogels were produced as described in section 3 and cylindrical micropatterned hydrogels (Ø 6mm) of PLMA at 15% w/v with both hexagonal and square arrangements were herein used for the cell culture assays. Figure 3 shows a schematic representation of the procedure for cell culture assays. Cells were seeded on top of micropatterned PLMA hydrogels and cultured during 4 days. Cell culture assays with HUVECS, H9c2(2-1) and a co-culture of both were performed. For the HUVECs and H9c2(2-1) assays a cell density of 1 x 10^5 cells was used per gel. For the co-culture assay HUVECs and H9c2(2-1) were cultured in a final proportion of 1:1 in a cell density of 5 x 10^4 cells per gel for each type of cell.

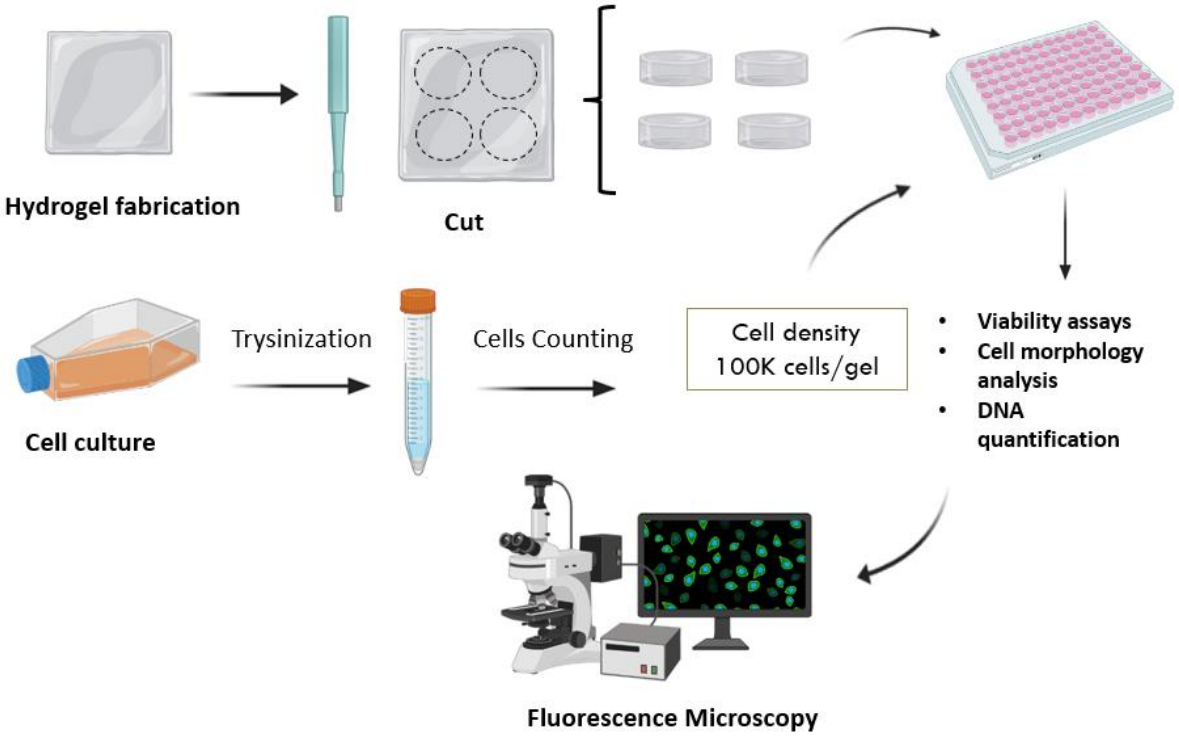

Figure III.3. Schematic illustration summarising cell culture assays procedure.

4.1. Cell viability by Live/Dead Assay

Live/dead was performed to assess cell viability cultured on top of the micropatterned PLMA hydrogels. At pre-determined time-points (1 and 4 days of culture), hydrogels were incubated in a solution of 2 μL of calcein-AM and 1 μL of propidium iodide (PI) 1 mg mL−1 (Thermo Fisher Scientific, USA) in 1000 μL of PBS at 37 °C during 30 min.
After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

PI is a fluorescent red chromosomal and nuclear counterstain that is widely used as it is impermeable to living cells and thus allows the detection of dead cells, giving them a red fluorescence. On the other hand, calcein-AM is used to test cell viability. This non-fluorescent dye, after hydrolysis of the acetoxymethyl ester by intracellular esterases, is converted into a fluorescent green calcein, thus allowing the detection of living cells.

4.2. Cell morphology analysis by DAPI/phalloidin staining

DAPI/phalloidin staining was made to analyse the morphology of the cells placed on the top of the hydrogels.

At pre-determined time points, hydrogels were washed with PBS and fixed with a 4% v/v formaldehyde (Sigma Aldrich, Germany) solution for at least 2 h. For DAPI/phalloidin staining, a phalloidin solution (Flash Phalloidin Red 594, 300U, BioLegend, USA) was diluted 1:40 in PBS and hydrogels were incubated at room temperature for 45 min. After washing with PBS, a DAPI (Thermo Fisher Scientific, USA) solution was diluted in 1:1000 PBS and hydrogels were incubated for 5 min at room temperature. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

DAPI (diamidino-2-phenylindole) is a blue fluorescent probe with high cell permeability that fluoresces brightly by specifically binding to the minor groove of double-stranded DNA. Its stain is used to label cell nuclei by fluorescence microscopy. Phalloidin is a bicyclic peptide that can be used as a fluorescent probe for cell labelling of actin filament structure. Thus, the filamentous F-actin structure of the cells presents a red fluorescence.

4.3. Cell proliferation by DNA Quantification

DNA quantification was performed using a Quant-iT PicoGreen dsDNA kit (Thermo Fisher Scientific, USA). At pre-determined time points, hydrogels were washed with PBS, incubated in sterile deionized water, and frozen at −80 °C until the tests were performed. The disruption of the cells placed on the top of the hydrogels was induced through their thaw at 37 °C and placed in an ultrasound bath for ≈30 min. DNA standards were prepared with concentrations ranging between 0 and 2 μg mL^{-1}. The plate was incubated for 10 minutes at room temperature in the dark and fluorescence was measured using an excitation of 480 nm and an emission of 528 nm (Synergy HTX, BioTek Instruments, USA).

PicoGreen's dsDNA quantification kit is an ultrasensitive fluorescent nucleic acid that allows to quantify double-stranded DNA (dsDNA), even in the presence of RNA, in molecular biological procedures such as, PCR-based assays, microarray samples, DNA damage assays, enzyme activity assays, genomic DNA quantitation, measuring dsDNA in complex mixtures, and viral DNA quantitation.

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Chapter IV

Micropatterned platelet lysate-based membranes for cardiac regeneration

Micropatterned platelet lysate-based membranes for cardiac regeneration

Abstract

Myocardial infarction (MI) is one of the most serious health problems, leading to the destruction of heart tissue and putting patients at constant risk of heart failure. Tissue engineering (TE) has emerged as an alternative to conventional pharmacological therapies or organ transplantation producing structures with potential for application in cardiac regeneration. The ideal 3D structure for cardiac TE must combine several structural and functional properties that should match that of the native myocardium.

Methacrylated platelet lysates (PLMA) based hydrogels have recently been studied as a human-based biomaterial with tuneable mechanical properties that support the cell culture of human cells. In this work, polydimethylsiloxane (PDMS) was used to produce molds with different topographies to be used as counter molds for PLMA-hydrogels fabrication through polymerization by UV light exposure. Hexagonal and square geometrical micropillars arrangements were the topographies used to produce micropatterned PLMA hydrogels. Afterwards, cell culture assays with human umbilical vein endothelial cells (HUVECs) and myoblasts derived from embryonic myocardium rat tissue (H9c2(2-1)) were performed in the micropatterned substrates. Results show that cells were able to adhere to the hydrogels and organize within the structures, maintaining their viability until 4 days in culture. Overall, the results demonstrate that micropatterned PLMA-hydrogels have suitable biomechanical properties, allowing a good cell adhesion, alignment and proliferation.

1. Introduction

Cardiovascular diseases are increasing all over the world, being myocardial infarction (MI) one of the most serious heart associated problems. MI is characterized by cell death in the myocardium due to prolonged ischemia, disabling this tissue to transmit electrical signals or contract properly, putting patients at constant risk of heart failure (HF).[1,2] Thus, the treatment of patients post-MI has been a great focus in scientific research community more

specifically in the field of tissue engineering and regenerative medicine (TERM), which seeks to overcome the disadvantages of conventional therapies by combining biomaterials, cells and biomolecular factors.[3]

Cell-based therapies for cardiac diseases, in particular cell injection in the damaged tissue, emerged as interesting alternatives to conventional pharmacological treatments and heart transplantation, allowing the repair of irreversible negative changes in the biomolecular environment and the mechanical properties of the damaged myocardial tissue area, taking advantage of its intrinsic regenerative capability.[4] This type of treatment was undoubtedly an excellent innovation in cardiac regeneration but it has some disadvantages such as extremely low cell retention and engraftment rate in the host myocardium, thus decreasing the effectiveness of regeneration.[5] Therefore, several studies in TERM field have been carried out regarding for example the development of novel platforms like hydrogels as an alternative to serve as support to deliver cells to damaged tissues. In addition to allowing the use of cells, hydrogels present several advantageous properties such as stiffness, toughness and adhesion capacity to obtain a microenvironment that mimics the native tissue.[6,7]

Cardiac patches have emerged as platforms that can be combined with cells and applied in the damaged area of the myocardium in order to improve the regeneration process. Biomechanical properties have been one of the focuses in this type of biomaterials, but in addition to the type of material used, surface topographies for cell culture have been increasingly developed. Topography in biomaterials has influence on cell mechanotransduction, allowing better alignment and organization of the tissue formed as well as cell-cell interactions that are important features in cardiac regeneration. In addition to a microenvironment conducive to cell adhesion and proliferation, it is intended to mimic the anisotropy of the native tissue.[8,9]

Hydrogels derived from methacryloyl platelet lysates (PLMA) have been recently reported. PLMA is obtained from platelet lysates (PLs) by modification with methacryloyl groups, making them photoresponsive. After modification, PLMA hydrogels can be produced by exposure to UV light. Such hydrogels have not only the mechanical strength but also the biocompatible properties required to support cell adhesion and proliferation.[10–13]

This project proposes the production of micropatterned PLMA-based hydrogels with two types of micropillars topography - hexagonal and square. The micropillars were formed by soft photolithography using polydimethylsiloxane (PDMS) micropatterned patches as counter molds, containing wells with dimensions of interest. The effect of the micropatterns on cell adhesion and function was studied and compared with surfaces without topography. Results suggest that the use of topographical cues have a positive influence in cell adhesion to PLMA surfaces when compared to non-patterned surfaces.

2. Materials and methods

2.1. Synthesis of Methacryloyl Platelet Lysates (PLMA)

Following a previously reported methodology[10], platelet lysates (PLs, STEMCELL Technologies, Canada) were thawed at 37 °C and to 10 mL (total protein content 6.3 g dL^{-1}) of this solution, 100 μL of methacrylic anhydride was added in order to synthesize PLMA. The reaction was carried out under constant stirring at room temperature. The synthesized PLMA was then purified by dialysis against deionised water to remove the excess of methacrylic anhydride. The PLMA solution was then filtered with a 0.2μm filter to sterilize it, frozen with liquid nitrogen, freeze-dried, and stored at 4°C until further use.

2.2. Preparation of PLMA hydrogels with different topographies

In this work, polydimethylsiloxane (PDMS) was used to produce molds with different topographies to be used as counter molds for hydrogels fabrication. PDMS is prepared by mixing the curing agent in a base polymer (Sylgard™ 184 Silicone Elastomer Kit, Dow Corning) following a 1:10 ratio. These two components are mixed and the final mixture is degassed in vacuum for 30 minutes to remove any remaining air bubbles and placed in an oven at 60ºC for 2h for curing.[14,15]

Two different PDMS micropatterned patches with microwells were prepared by a double cast moulding process according to a protocol previously reported[16]: one with a hexagonal arrangement and another with a square arrangement. For both arrangements, the microwells have a depth of 100 µm, diameter of 60 µm but different spacing between them: 40 µm and 80 µm.

To produce the PLMA-based hydrogels with different topographies lyophilized PLMA was dissolved in a solution of 0.5% w/v Irgacure 2959 (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (Sigma-Aldrich, Germany) in phosphate-buffered saline (PBS, Thermo Fischer Scientific, USA) to final concentrations of 10%, 15%, or 20% w/v of PLMA. Afterwards, PLMA hydrogels were made by pipetting the final solution to a PDMS mold and the micropatterned patch previously mentioned was placed above the PLMA solution followed by UV exposure (0.95 W cm^{-2}) for 10 min.

To help PDMS patch detachment from PLMA hydrogels and to maintain the micropillar structure, the PDMS micropatterned patches were washed with ethanol, dried, and finally taken to a hydrophilic treatment in a ATTO low-pressure plasma system (Diener) for 7 min (70V, 0.4-0.6 mbar) prior to the fabrication of the hydrogel.

After detachment of the PDMS micropatterned mold, PLMA hydrogels with micropillar are obtained. These hydrogels have a square shape with an area of 1 cm^2, but afterwards four circles with a diameter of 6mm are cut with a biopsy punch (Kai Medical, Japan).

2.3. PLMA micropatterned hydrogels characterization – Mechanical properties

Mechanical assays were performed based on compression tests employing an Instron Universal Mechanical Testing Machine 3343 (Instron, USA) equipped with a load cell of 50 N. The cylindrical hydrogels specimens, prepared the day before and placed in PBS overnight, were tested at room temperature. For each hydrogel, the displacement data from initial until rupture load were measured using a computer data acquisition system connected to the tester (Bluehill® Universal, Instron).

The Young's modulus was defined as the slope of the linear region of the strain–stress curve, corresponding to 0–5% strain. Ultimate stress and ultimate strain values were taken as the point where the failure of the hydrogel occurred.

2.4. In vitro cell culture of cardiomyoblasts and endothelial cells

Cell culture assays on micropatterned PLMA hydrogels were performed with human umbilical vein endothelial cells (HUVECs, ATCC) and myoblasts derived from embryonic myocardium rat tissue (H9c2(2-1), ATCC).

HUVECs were cultured in Medium 199 (Sigma-Aldrich) supplemented with 20% FBS, 1% antibiotic/antimicotic, 1% of glutamate, 1% v/v of Heparin (100 mg/mL, Sigma-Aldrich), and 0.1% v/v of Endothelial Cell Growth Supplement (40 mg/mL, Merck, Germany). HUVECs were used until passage 8. Cell suspensions were prepared by trypsinization with a trypsin/EDTA solution (Sigma-Aldrich, Germany). H9c2(2-1) were cultured in Dulbecco's Modified Eagle Medium High Glucose (DMEM-HG, Sigma-Aldrich) supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic/antimycotic. Cell suspensions were prepared by trypsinization with a TrypLE™ Express solution (Thermo Fisher Scientific).

Micropatterned PLMA hydrogels were produced as described in section 2.2 and cylindrical micropatterned hydrogels (Ø 6mm) of PLMA at 15% w/v with both hexagonal and square arrangements were herein used for the cell culture assays. For the HUVECs and H9c2(2-1) assays a cell density of 1 x 10^5 cells was used per gel. For the co-culture assay HUVECs and H9c2(2-1) were cultured in a final proportion of 1:1 in a cell density of 5 x 10^4 cells per gel for each type of cell. As control, PLMA hydrogels without micropatterning were used.

2.4.1. Cell viability by Live/Dead assay

Live/dead was performed to assess viability of cells cultured on top of the PLMA hydrogels. At pre-determined time-points, hydrogels were incubated in a solution of 2 μL of calcein-AM and 1 μL of propidium iodide (PI) 1 mg mL^{-1} (Thermo Fisher Scientific, USA) in 1000 μL of PBS at 37 °C during 30 min. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

2.4.2. Cell morphology analysis by DAPI/phalloidin staining

DAPI/phalloidin staining was performed to analyse the morphology of the cells placed on the top of the hydrogels. At pre-determined time points, hydrogels were washed with PBS and fixed with a 4% v/v formaldehyde (Sigma Aldrich, Germany) solution for at least 2h. For DAPI/phalloidin staining, a phalloidin solution (Flash Phalloidin Red 594, 300U, BioLegend, USA) was diluted 1:40 in PBS and hydrogels were incubated at room temperature for 45 min. After washing with PBS, a DAPI (Thermo Fisher Scientific, USA) solution was diluted in 1:1000 in PBS and hydrogels were incubated for 5 min at room temperature. After washing with PBS, hydrogels were examined using a fluorescence microscope (Fluorescence Microscope Zeiss, Axio Imager 2, Zeiss, Germany).

2.4.3. Cell proliferation by DNA quantification

DNA quantification was performed using a Quant-iT PicoGreen dsDNA kit (Thermo Fisher Scientific, USA). At pre-determined time points, hydrogels were washed with PBS, incubated in sterile deionized water, and frozen at −80 °C until the tests were performed. The disruption of the cells placed on the top of the hydrogels was induced through their thaw at 37 °C and placed in an ultrasound bath for ≈30 min. DNA standards were prepared with concentrations ranging between 0 and 2 μg mL^{-1}. The plate was incubated for 10 minutes at room temperature in the dark and fluorescence was measured using an excitation of 480 nm and an emission of 528 nm (Synergy HTX, BioTek Instruments, USA).

2.5. Statistical analysis

All data were statistically analysed using the GraphPad Prism 7 software and were reported as mean ± standard deviation. Statistical differences were determined using two-way ANOVA analysis with Tukey's multiple comparison test.

Results and Discussion

1. Methacryloyl platelet lysates (PLMA) synthesis

Platelet lysates (PLs) are a source of proteins and growth factors (GFs), important molecules involved in cell recruitment, growth and morphogenesis, thus being a material with great potential for use in tissue regeneration strategies.[17,18]

In this work, PLs were modified with methacryloyl groups to make them photoresponsive (PLMA). For the modification, PLs were reacted with methacrylic anhydride at room temperature in a 100:1 ratio. This modification allows the insertion of functional groups in the proteins' backbone - methacryloyl groups - causing PLs to become photoresponsive. Figure 1 shows a schematic representation of the process to produce PLMA.

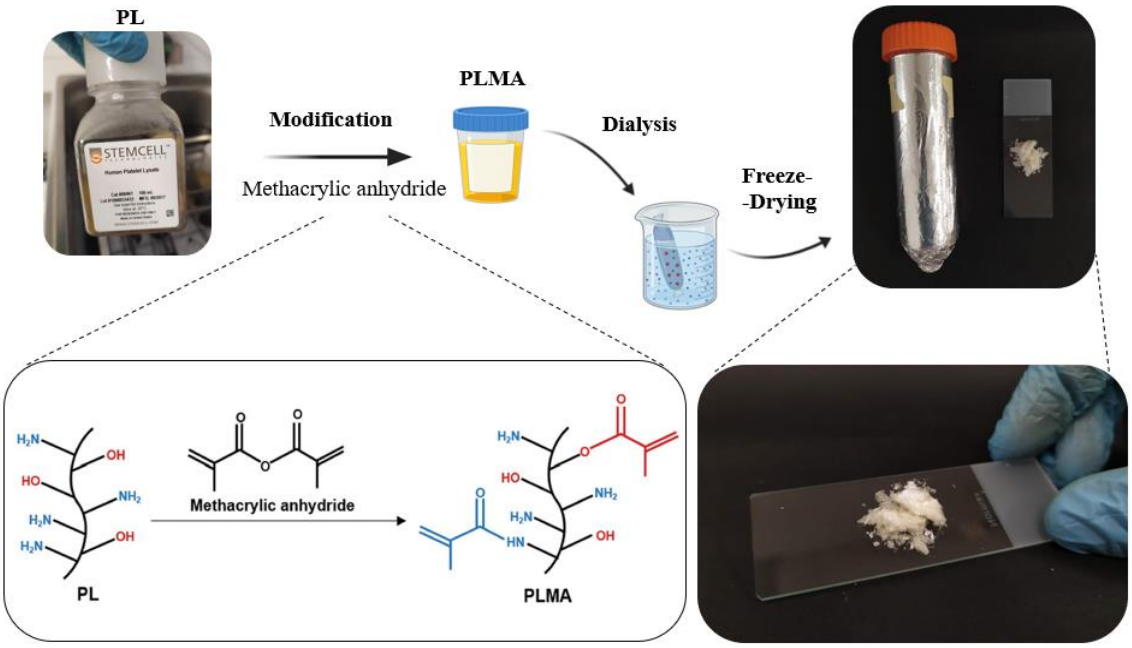

Figure IV.1. Schematic representation of PLMA synthesis process. Chemical reaction's image adapted from [10].

2. Micropatterned PLMA hydrogels production

In this work, PDMS molds with four different microwells arrangement were used (see figure 2): (a) hexagonal and (b) square, both with a microwells spacing of 80 µm and (c) hexagonal and (d) square, both with microwells spacing of 40 µm. From the PDMS counter molds, the micropillars structure was obtained (figures 2e and 2f). For all condition the micropillar height was 100 µm and the micropillar diameter was 60 µm.

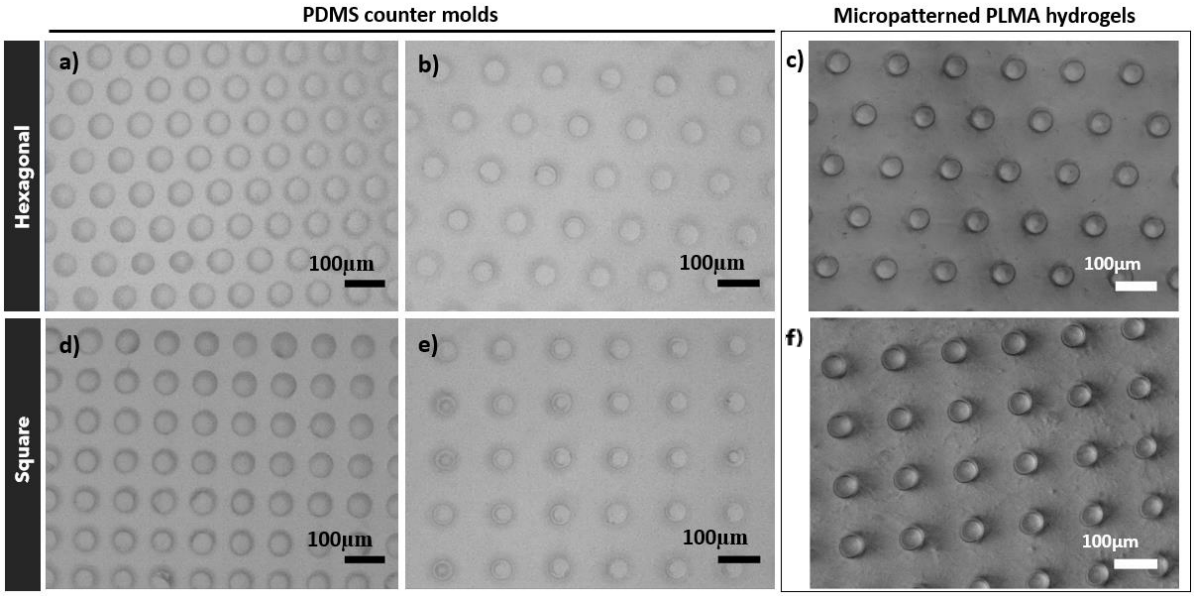

Figure IV.2. Representative brightfield images of the four different topographies explored in this work: (a) hexagonal and (b) square, both with a microwells spacing of 80 µm and (d) hexagonal and (e) square, both with microwells spacing of 40 µm, all of them with microwell depth of 100 µm and diameter of 60 µm; and brightfield images of the micropillars structure present in micropatterned PLMA-hydrogels (c and f).

The brightfield images of the micropatterned surface in PLMA-hydrogels (figures 3c and 3f) show well-defined micropillars, which represents a good reticulation of the PLMA solution during photopolymerization by UV exposure. It is possible to notice that the micropattern is quite uniform and regular, maintaining the intended established structure.

3. Mechanical properties of micropatterned PLMA hydrogels

PLMA-based hydrogels at 10, 15 and 20% (w/v) were the three conditions under analysis to study their mechanical properties. Hydrogels with the two different topographies – hexagonal and square – were herein analysed to assess the mechanical properties of this biomaterial by means of compression tests (figure 3A).

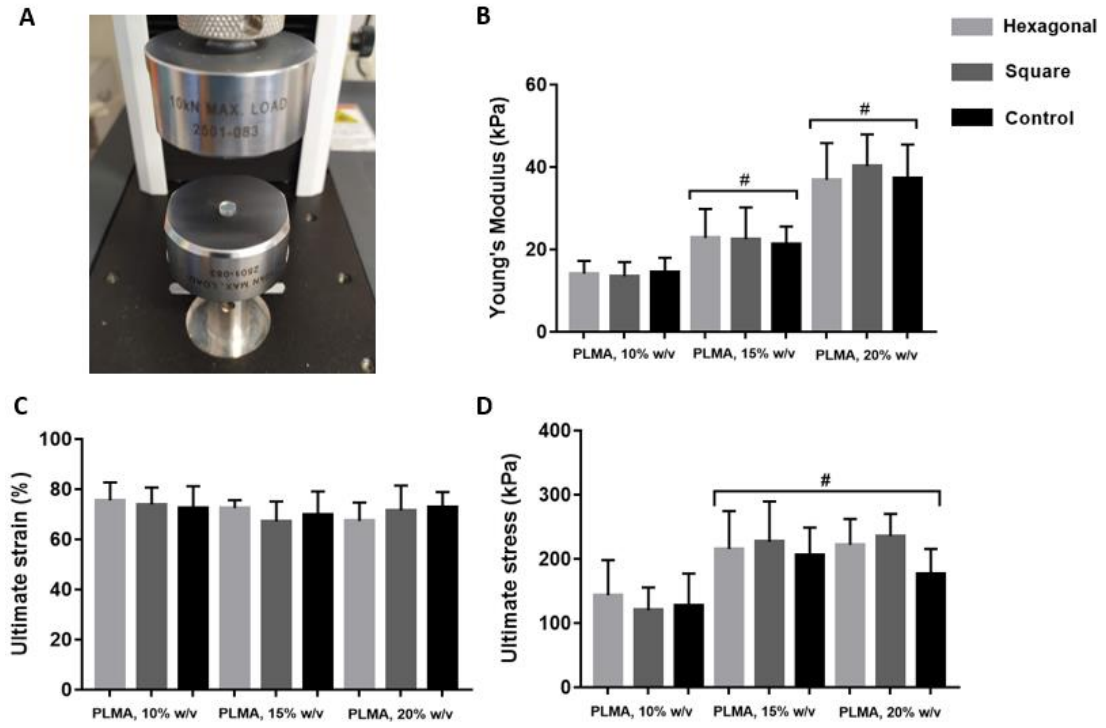

Figure IV.3. (A) Mechanical testing by compression tests of micropatterned PLMA hydrogels. (B) Young's modulus (kPa), (C) Ultimate strain (%) and (D) Ultimate stress (kPa) of micropatterned PLMA-based hydrogels for the conditions of 10, 15 and 20% w/v hydrogels. Statistical differences between the analysed groups (#, significantly different from all the others on the left) were determined using two-way ANOVA analysis with Tukey's multiple comparison test.

In addition to properties such as biocompatibility and biodegradability, scaffolds are intended to have suitable mechanical properties to mimic the native tissue and be a good support for cell culture.[19,20] PLMA-based hydrogels, in comparison with other previously studied PLs-derived materials, have been shown to have tuneable mechanical properties and adequate stability for *in vitro* cell culture.[10] It is known that the myocardial tissue possesses contractile movements that require, a range of elasticity to support the compression forces

exerted by the heart. Thus, compression tests were carried out to obtain the elasticity values for each condition of PLMA-based hydrogel. The Young's modulus, or also called elastic modulus, allows to understand the behaviour of a material under tension or compression conditions, thus measuring the degree of stiffness.

For all the tested conditions, the Young's modulus (figure 3B), increase according to the increase of PLMA concentration used for the manufacture of each hydrogel, being the hydrogels at 20% the stiffest one, with PLMA hydrogels at conditions 10 and 15% having an Young's modulus closer to the native cardiac tissue (10-15 kPa).[10,21]

While the Young's modulus allows us to understand the stiffness of the PLMA hydrogels, the values of ultimate stress and ultimate strain represent the maximum force of deformation that the material can support until the break point. In figure 4C we can see that independently of the topography analysed, the deformation capacity is similar, with no significant differences in the ultimate strain values between conditions. However, the ultimate stress values (figure 3D) show the higher compressive strength of PLMA hydrogels at 15 and 20% w/v conditions.

Considering the results from mechanical analysis, the hydrogels used for cell culture studies were those of the 15% w/v condition due to their stiffness closer to that found in native myocardial tissue, being also mechanically more resistant than the PLMA hydrogels at 10% w/v.

4. In Vitro Cell Culture

According to the literature, cells tend to respond to surface topography by means of a contact guide, leading to changes in the organization of the cytoskeleton and cell migration according to the pattern provided. In this work, micropatterns with different micropillars arrangements in the surface of PLMA hydrogels were studied in order to understand how it affects the adhesion and alignment of cells. For the cell culture assays, endothelial cells - HUVECs – and cardiomyoblasts – H9c2(2-1) – were seeded on top of micropatterned PLMA hydrogels with both hexagonal and square arrangement and in unpatterned hydrogels which serve as a control.

For each cell type, different spacing between the micropillars existing in the produced

PLMA-hydrogels were tested. It is known that the dimensions of human ventricular CMs are 100-150 μm by 20-35 μm, which demonstrates a great capacity for morphological elongation of this type of cell.[22] Thus the higher micropillar spacing (80 μm) was used for H9C2 culture. The average dimensions of the ECs are approximately 50-70 μm in length and 10-30 μm in width. Although ECs morphology and size vary with the type and diameter of the arteries due to factors such as the effect of blood flow direction and volume rate. Thus the smaller micropillar spacing (40 μm) was used for HUVECs culture.[23]

4.1. Cell viability, morphology, and proliferation assays

4.1.1. Human umbilical vein endothelial cells culture

Human umbilical vein endothelial cells (HUVECs) were used with the aim of mimicking the vascularization of cardiac tissue, a key factor in promoting angiogenesis. According to the literature, ECs represent the largest percentage of non-myocytes, having great therapeutic importance, corresponding to a large part to vascularization, and a small part to lymphatic vessels, which also proves evidence of having an important role in cardiac regeneration.[24]

HUVECs were seeded on the micropatterned surface of the hydrogel, and the culture was performed during 4 days. At pre-determined time points (1 and 4 days) a live/dead staining was performed, and results show that ECs have a tendency to surround the micropillars present in the hydrogels in both hexagonal and square arrangements – see figure 4A. In addition, a DAPI/Phalloidin staining (figure 4B) was performed to analyse cell morphology. This assay confirmed the elongation of the cell structure around the micropillars present on the surface of micropatterned PLMA hydrogels.

Overall, cells show a tendency to migrate to areas around the micropillars in both arrangements. In fact, previous studies show better adhesion of ECs in flatter regions of created topographies.[25,26] This process is called mechanotransduction and involves the cells' reaction to mechanical stimuli such as this topography.[27,28] In this cellular process, the biophysical properties of the microenvironment are converted into biochemical signals, involving several pathways through integrins, focal adhesions and actin cytoskeleton that alter cell behaviour.[29–31]

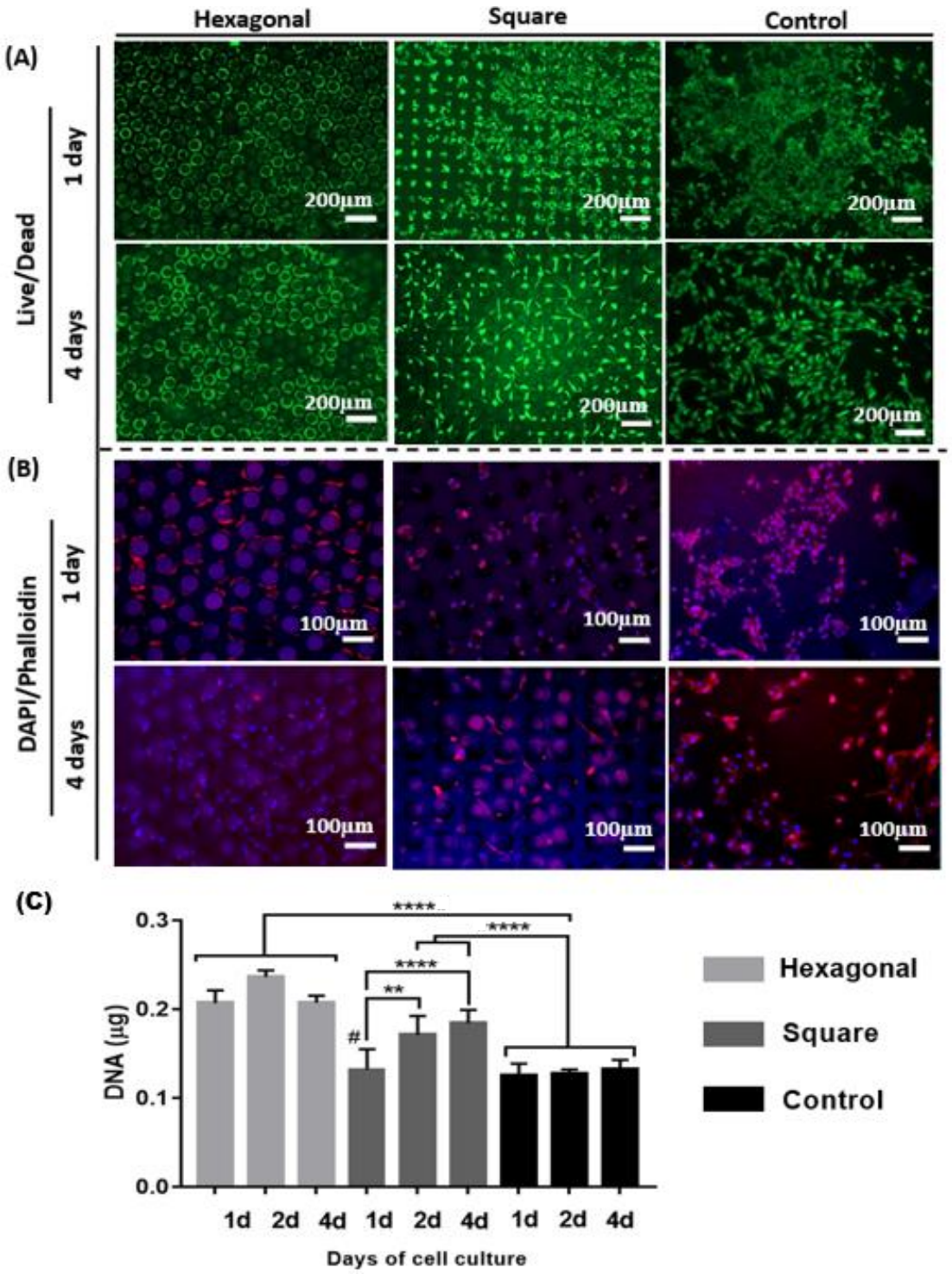

Figure IV.4. Fluorescence microscopy images of (A) live/dead (Scale bar: 200µm) and (B) DAPI/phalloidin (Scale bar: 100µm) staining of HUVECs cultured on the top of micropatterned PLMA-based hydrogels and unpatterned PLMA-based hydrogels (control) at 1 and 4 days of culture; and (C) DNA results at 1 , 2 and 4 days of cell culture. Statistical differences (**P < 0.01; ****P<0.0001) between the analysed groups (#, significantly different from all the others on the left) were determined using two-way ANOVA analysis with Tukey's multiple comparison test.

Both the live/dead and DAPI/Phalloidin images also show how the absence of topography (as in the case of the control sample) does not affect the direction of the cells, which can be observed by the randomness of their adhesion in the hydrogel. In the hexagonal pattern, the elongation of the cells around the micropillars is clearly verified, while in the square pattern, in addition to this elongation, the beginning of interactions between cells is observed.

HUVECs were able to proliferate on the micropatterned PLMA-hydrogels as can be seen by the increase in DNA content (Figure 4C), specially in square micropillar arrangement which confirms the good adhesion and initial cell-cell communication observed in fluorescence images of viability assays (Figure 4A and 4B). The PLMA-hydrogel with hexagonal pattern, despite showing a decrease in DNA concentration values from day 2 to day 4 of cell culture, remains identical to that existing on the day 1 of culture.

4.1.2. H9c2(2-1) cell culture

H9c2(2-1) were seeded on the micropatterned surface of the hydrogel, and the culture assay was performed during 4 days. Live/dead tests were performed at 1 and 4 days of culture and results show that cardiomyoblasts were able to adhere to micropatterned PLMA-hydrogels and maintain their viability until 4 days of culture – see figure 5A. As well, DAPI/Phalloidin staining (Figure 5B) showed the ability of these cells to adhere to the micropatterned PLMA hydrogels, exhibiting elongated morphology after 4 days and demonstrating a cell network orientation accordingly to the micropillars.

According to the literature, the cooperation between CMs in the human heart positively influences the growth and remodelling of myocardial tissue.[32] After a myocardial infarction, the tissue have an enormous lack of cells, especially CMs.[33] To mimic the native cardiac microenvironment, a biomaterial must have the necessary mechanical structure to allow CMs remodelling and alignment, important characteristics to take into account for a future-clinical application for cardiac tissue regeneration.[34] By observing the images obtained in the live/dead assays, this objective is fulfilled since the H9c2(2-1) demonstrated good adhesion to the micropillars and a good cellular association at hexagonal and square patterns which reflects the so-called contact guidance, as we can affirm that the topography applied in PLMA influenced the cellular behavior.[35]

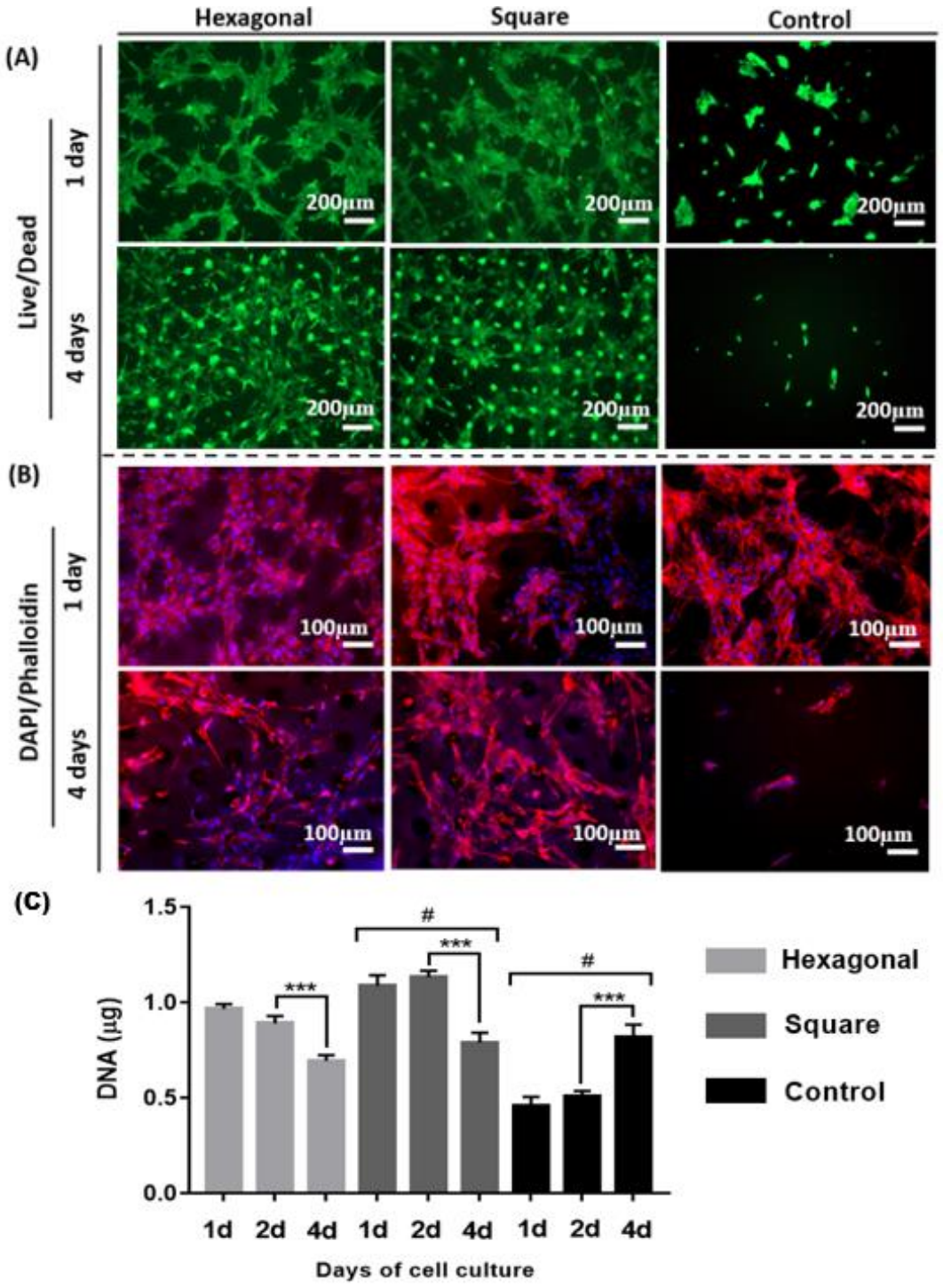

Figure IV.5. Fluorescence microscopy images of (A) live/dead (Scale bar: 200µm) and (B) DAPI/phalloidin (Scale bar: 100µm) staining of H9c2(2-1) cultured on top of micropatterned PLMA-based hydrogels and unpatterned PLMA-based hydrogels (control) at 1 and 4 days of culture; and (C) DNA results at 1, 2 and 4 days of cell culture. Statistical differences between the analysed groups (#, significantly different from all the others) were determined using two-way ANOVA analysis with Tukey's multiple comparison test. (***P<0.001)

Cell viability assays show that there is a pre-alignment of cardiomyoblasts along the micropillars, both geometries being very promising for the construction of a microtissue with potential for application in cardiac regeneration. It is noteworthy that there is a significant difference between cell viability in patterned PLMA-hydrogels compared to unpatterned ones, with a much greater adherence when topography is applied to the hydrogel. Indeed, on day 4 of culture, the cardiomyoblasts, despite not showing a unidirectional proliferation, form a dense cellular network that demonstrate a good interaction between cells. The DAPI/Phalloidin morphology assays confirm the above discussed results by verifying a great elongation of the cells in the micropillars. On day 1 of cardiomyoblast culture, the unpatterned PLMA-hydrogel, by staining with phalloidin, demonstrated good structure of actin filaments (see in figure 5B), showing relatively good adhesion, but on day 4 the cells no longer have adequate proliferation for use for mimic tissue, which proves how topography brings considerable improvements in cell behaviour and survival. In this way we can state that it was possible to create an initial phase of microtissue formation with good cell communication for both PLMA-hydrogels with hexagonal and square patterns, which indicates an adequate profile in mimicking the native tissue, once again presenting themselves as good platforms to cellular alignment, orientation, and cell-cell interactions.In figure 5C it can be seen a significantly decrease of the DNA content of H9c2(2-1) cultured on the top of patterned PLMA-hydrogels after 4 days of culture. Such differences can be explained by the fact that when cell culture time increases it is more difficult to perform cell lysis cultured in PLMA hydrogels. As such, increasing the freeze-thawing cycles to increase cell disruption can be a way to have DNA results that better corroborate live/dead assays.

Conclusions and Future Perspectives

In this thesis a novel concept of human native tissue cardiac-mimicking scaffold is proposed. The use of modified platelet lysates (PLMA) to fabricate hydrogels shows increased and easily adjusted mechanical properties. PLMA is an alternative xeno-free material for scaffold fabrication which allows cell culture with interesting results as previously mentioned. The PLMA-hydrogels' tuneable mechanical properties are reached by adjusting the concentration, which was demonstrated by the results presented, where the

stiffness of PLMA-hydrogels increased with the increase of the concentration of material used. PLMA-hydrogels at 15% w/v, compared to other studied conditions, show to have suitable mechanical properties closer to the existing at native cardiac tissue.

PLMA-hydrogels support the adhesion and proliferation of the two types of cells used – HUVECs and H9c2(2-1) – with good results for hexagonal and square patterns used. In this work, it is possible to confirm the influence of topography on the orientation of the cells, which demonstrates that the existing micropillars on top of the hydrogels provide a suitable contact guidance for communication between cells.

There is now ongoing work in the optimization of the parameters to culture both cell types and identify the geometry and spacing between pillars that results in better cell alignment and proliferation. Future work will be performed to identify the ideal conditions for co-culturing both cell types. Nevertheless, PLMA-hydrogels can be seen as potential platforms for clinical application to repair a injured tissue post-MI.

The investigation about these novel micropatterned PLMA-based membranes just started and have a lot of possible research to enrich the results confirmation about the great potential of this platform for cell culture and cardiac tissue regeneration.

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Chapter V

Conclusions and Future Perspectives

Conclusions and Future Perspectives

Heart tissue has self-renewal capacity to regenerate the injured myocardium tissue post-myocardial infarction (MI). This leads to the formation of a scar tissue in the heart with poor electrical and contraction properties, putting patients at risk of subsequent heart failure (HF). To date, the only viable option for patients with the end-stage heart disease is whole heart transplantation. However, the shortage of donor hearts makes this approach unavailable for most of patients. The development of new and effective techniques for regenerating injured myocardium would thus have important therapeutic implications.

The field of cardiac tissue engineering (TE) has evolved as a promising and actively developing area of research that aims to repair, replace, and regenerate the myocardium. Cell-based strategies have shown potential to decrease scar formation and enhance heart contractility. The cells release bioactive factors that help restore damaged tissue, although, the technique has so far shown inconsistent results. A bigger challenge in cardiac repair is to increase cell engraftment and assembling the cells into functional tissue. This may be achieved by using injectable materials as cell carriers or seeding cells into scaffolds and patching them onto the heart.

Platelet-lysates (PLs) are a complex mixture of multiple proteins, growth factors and cytokines which are bioactive molecules involved in cell recruitment, growth, and morphogenesis, essential properties that make this human-derived material promissor for tissue regeneration. In this work, PLs were used to produce methacryloyl platelet lysates (PLMA) by reaction with methacrylic anhydride. This modification allows chemical crosslinking of PLMA by exposure to light leading to hydrogel formation.

The main aim of this project was the development of PLMA-based hydrogel membranes with topographic features produced by soft photolithography. Such hydrogels were used to culture both endothelial and cardiac cells in order to understand how cells behave in the micropatterned scaffolds. In this work, PLMA-based hydrogels with micropillars were produced with two different pillars arrangements – hexagonal and square. It was demonstrated that the hydrogels have tunable mechanical properties adjusted by the concentration of the polymer in the precursor solution and good stability *in vitro*, being that key features to have a scaffold that could be used to mimic the heart microenvironment. Cellular assays demonstrated good adhesion and proliferation for both types of cells used –

human umbilical vein endothelial cells (HUVECs) and myoblasts derived from embryonic myocardium rat tissue (H9c2(2-1)) – regardless micropillars arrangement. It was also possible to confirm that the presence of the micropattern influence cell orientation. Although these results show the ability of micropatterned PLMA-based hydrogels to be used as platforms for the culture of endothelial cells and cardiomyoblasts, in the future it would be interesting to perform cell culture assays during more time to better understand how the micropillar arrangements can influence cell proliferation. It would also be interesting to perform cell culture assays in micropatterned PLMA-hydrogels with different micropillar spacing in order to observe its influence in cell behaviour.