POLITECNICO DI TORINO

SCUOLA INTERPOLITECNICA DI DOTTORATO

Doctoral Program in Biomedical and Biomechanical Engineering

Final Dissertation

# Cardiac tissue engineering: designing innovative approaches combining bioreactor-based technologies and biological strategies

**Giulia Cerino Abdin**

Tutors

Prof. Cristina Bignardi
Dr. Diana Massai

Coordinator of the Research Doctorate Course

Prof. Cristina Bignardi

2011 – 2013

## Abstract

Heart disease is the leading cause of morbidity and mortality in the Western World, claiming 7.25 million deaths every year, with an increasing trend destined to rise up to about 23.6 million in 2030. This scenario greatly motivates research into effective therapeutic interventions, since complete native regeneration is unlikely for fully differentiated, load-bearing cardiac tissues.

However, current therapeutic cardiac strategies, e.g., transplantation of autologous and donor grafts, and implantation of artificial prostheses or devices as left ventricular assist devices, suffer from limitations. The principal contraindications in using autologous grafts are identified in donor site morbidity, limited availability, risk of infection, and secondary surgical wounds. In case of donor grafts, the disadvantages are the shortage of available donors, the risk of pathogen transfer and rejection, and mandatory lifelong immunosuppressive therapies. Finally, the use of artificial prostheses or devices implies limited durability, inability to completely restore natural functions, and often leads to the establishment of unphysiological conditions with the need of lifelong anticoagulation therapies.

Therefore, the innovative field of cardiac tissue engineering (TE) could represent an effective alternative to overcome the limitations of the current clinical therapies. This strategy, in fact, has shown the potential to generate both functional cardiac tissue substitutes for use in the failing heart, and biological *in vitro* model systems to investigate the cardiac tissue-specific development and diseases, allowing to perform accurate and controlled *in vitro* tests for cell and tissue-based therapies, drug screening, predictive toxicology and target validation. In the last decade, a great deal of progress was made in this field due to new advances in interdisciplinary areas such as biology, genetic engineering, biomaterials, polymer science, bioreactor engineering, and stem cell biology.

Each of these areas has contributed to the development of the three main components of the cardiac TE, i.e., cells, scaffolds and culture environment, that can be used individually or in combination: (1) cells synthesize the new tissue; (2) scaffolds provide physical support to cells and a structural and biochemical cue tailored to promote cell adhesion, migration, proliferation and differentiation; (3) biomimetic *in vitro* culture environments, designed to replicate the *in vivo* milieu by using biologically inspired requirements, influence and drive cells to differentiate towards the desired phenotype and to express their functions, promoting extracellular matrix formation and tissue maturation.

The objectives of this work concern two complementary aspects of the cardiac TE field, combining innovative bioreactor-based technologies and advanced biological strategies. In particular, during the PhD as engineering approach two different bioreactor prototypes for cardiac TE have been developed (Chapter II and III), while as biological and

applicative approach the work dealt with the generation of a contractile cardiac patch by using a perfusion-based bioreactor culture system (Chapter IV).

Bioreactors, technological devices designed for monitoring and controlling the culture environment and physiologically stimulating the construct, have become essential support tools in cardiac TE research.

Therefore, the first part of the research activity was dedicated to the development of two different bioreactor prototypes in the effort to replicate the native physical microenvironment of the cardiac tissue. Starting from a wide and in-depth review of the state of the art of bioreactors for cardiac TE (Chapter I), two bioreactor prototypes were developed (Chapter II and III), firstly, as model systems to investigate *in vitro* the effects of biophysical stimuli on cardiac cell culture and cardiac tissue formation and maturation. Secondly, once optimal culture conditions have been identified, bioreactors can be used as production systems for *in vitro* generation of functional engineered cardiac tissues. In detail, they will be illustrated: (1) a bioreactor for culturing cell-seeded cardiac patches, designed for generating a physiological biochemical and physical environment that mimics the native stimuli of the cardiac tissue, by providing uniaxial cyclic stretching and electrical stimulations (Chapter II); and (2) an innovative and low-cost bioreactor that, with peculiar geometric features, assures the possibility for buoyant vortices to be generate without using electromechanical rotating systems, allowing the establishment of suspension and low-shear conditions (Chapter III). For both the prototypes, preliminary cellular tests demonstrated the suitability of the devices for cardiac TE applications.

The second part of the research activity (Chapter IV) concerned a biological approach, with the aim to provide proof-of-principle for engineering reproducible thick-relevant skeletal myoblast-based contractile three-dimensional patches by using perfusion-based bioreactor culture system and type I collagen scaffold.

The research activities carried out in this thesis confirm the importance of combining complementary fields such as engineering and biology. A comprehensive approach that joins innovative bioreactor-based technologies with advanced biological strategies could provide an useful model system for cardiac development in the next future, while, in the late future, such approach could represent an effective therapeutic strategy to the reestablishment of the structure and function of injured cardiac tissues in the clinical practice, strongly contributing to the improvement of quality of life.

## Table of contents

## List of Abbreviations

| | |
|---|---|
| 2D | Two-Dimensional |
| 3D | Three-Dimensional |
| AV | AtrioVentricular valve |
| BCT | Bioartificial Cardiac Tissue |
| BEHM | Bioengineered Heart Muscle |
| BM-MSC | Bone-Marrow Mesenchymal Stem Cell |
| BPM | Beats Per Minute |
| CFD | Computational Fluid Dynamic |
| CMT | Cardiac MicroTissue |
| CM | Cardiomyocyte |
| $CO_2$ | Carbon Dioxide |
| CPE | Constant-Phase Element |
| DC | Direct Current |
| EC | Endothelial Cell |
| ECC | Engineered Cardiac Constructs |
| ECM | ExtraCellular Matrix |
| $EGFP^{pos}$ CPC | Enhanced Green Fluorescent Protein Cardiac Progenitor Cell |
| EPR | Ethylene Propylene Rubber |
| ES | Electrical Stimulation |
| ESC | Embryonic Stem Cell |
| ETH | Engineered Heart Tissue |
| FB | Fibroblast |
| FBMEs | Fibrin Based Mini Engineered Heart Tissue |
| FEM | Finite Element Method |
| GLP | Good Laboratory Practice |
| GMP | Good Manufacturing Practice |
| HARV | High Aspect Ratio Vessel |
| hETH | Human Engineered Heart Tissue |
| HSC | Hematopoietic Stem Cell |
| IGF-1 | Insuline-like Growth Factor 1 |
| IOD | Integrated Optical Density |
| iPSC | Induced Pluripotent Stem Cell |
| MEMS | Micro Electro-Mechanical System |
| MI | Myocardial Infarction |
| MSC | Mesenchymal Stem Cell |
| $O_2$ | Oxygen |

| | |
|---|---|
| PBS | Phosphate Buffered Saline |
| PC | PolyCarbonate |
| $pCO_2$ | Partial Pressure of Carbon Dioxide |
| PDMS | PolyDiMethylSiloxane |
| PFA | ParaFormAldehyde |
| PGA | Poly-Glycolic Acid |
| PGS | Poly-Glycerol Sebacate |
| PLA | Poly-Lactid Acid |
| PMMA | Poly-Methyl-MethAcrylate |
| $pO_2$ | Partial Pressure of Oxygen |
| POM | PolyOxyMethylene |
| PU | PolyUrethane |
| PVDF | PolyVinyliDene Fluoride |
| RPM | Revolution Per Minute |
| RWV | Rotating-Wall Vessel |
| SC | Stem Cell |
| SIS | Small Intestinal Submucosa |
| SMC | Smooth Muscle Cell |
| SM | Skeletal Myoblast |
| sPGA | Surface-hydrolyzed Ply-Glycolic Acid |
| STLV | Slow Turning Lateral Vessel |
| SVF | Stromal Vascular Fraction |
| TE | Tissue Engineering |
| VF | Volume Fraction |

# CHAPTER I

## Introduction

## 1. Clinical motivations

Cardiac disease is the leading cause of morbidity and mortality in the Western World (Ptaszek et al., 2012; Roger and Go, 2012). The inability of fully differentiated, load-bearing cardiac tissues to *in vivo* regenerate, and the limitations of the current treatment therapies greatly motivate the urgent demand for more efficacious pharmaceuticals to treat heart disease and new methods to repair damaged cardiac tissue. Novel approaches being developed to treat heart disease include cell and gene therapy, and cardiac tissue engineering (TE) strategies (Carrier, 2000). Cell therapy, due to the inability of adult cardiomyocytes (CMs) to proliferate and regenerate injured myocardium, has emerged as an alternative treatment option. Differentiated CMs are indeed an ideal cell source for injection, since they contain a developed contractile apparatus and can integrate through gap junctions and intercalated discs with the host CMs (Radisic and Sefton, 2011). However, large numbers of clinically relevant autologous CMs are unavailable. Other alternatives for appropriate cell sources, useful for the regeneration of infarcted myocardium, have been tested in animal models by transplantation of skeletal myoblasts (SMs) (Dorfman et al., 1998), as well as CMs derived from embryonic stem cells (ESCs) (Klug et al., 1996), and bone marrow-derived mesenchymal stem cells (BM-MSCs) (Toma et al., 2002). On the other hand, gene therapy approaches based on either delivering exogenous genes capable of expressing therapeutic proteins (Losordo et al., 1998; Miao et al., 2000), or on the blockade of genes involved in the pathological process (Akhtar et al., 2000; Mann and Dzau, 2000;), are becoming alternative strategies.

However, current cell and gene therapeutic cardiac strategies suffer from limitations. Cell therapy involves the intramyocardial or coronary injection of cells suspended in an appropriate liquid (saline or culture medium). The main challenges associated with this procedure are poor survival of the injected cells (Müller-Ehmsen et al., 2002a) and washout from the injection site (Reffelmann and Kloner, 2003). According to some estimates, ~ 90% of the cells delivered through a needle leak out of the injection site (Müller-Ehmsen et al., 2002a; 2002b). In addition, a significant number of cells die within days after injection (Zhang et al., 2001; Müller-Ehmsen et al., 2002a). Thus, developing improved delivery and localization methods (e.g. hydrogels) and effective anti-death strategies could significantly improve effectiveness of cell injection procedures. Similarly, gene therapy approaches for myocardial infarction (MI), especially acute MI, are limited by the available delivery techniques. In general, in fact, the time it takes for transcription and translation is too long for a successful intervention in acute MI (Melo et al., 2004). Moreover, targeting a single gene as most commonly used in gene therapy may have conceptual limitations as well. Most pathological processes are complex and involve expression or downregulation of multiple genes. In many instances, this genetic complexity is not well understood and thus is difficult to predict a priori what the ultimate effect of

overexpressing or blockade of a single gene will be. In this respect, combination of gene and cell therapy may be a preferred approach in the treatment of heart diseases (Radisic and Sefton, 2011).

Alternatively, the innovative field of cardiac TE, aiming to generate cardiac muscle cell-constructs to be used as three-dimensional (3D) models for *in vitro* physiological and pharmacological studies and eventually for repair of damaged heart muscle *in vivo* (Carrier, 2000), could represent an effective alternative to overcome the current clinical limitations. Attempts to *in vivo* stimulate the regeneration of injured cardiac tissues were pursued on animal models by: (1) injection of differentiated cells or stem cells (SCs) in situ (Hassink et al., 2003a; 2003b); (2) mobilization of endogenous SCs with cytokines (Orlic et al, 2001); (3) activation of CM cell cycle (Pasumarthi and Field, 2002; Field, 2004) obtained, e.g., by inducing permanent coronary artery occlusion (Hassink et al., 2008) or performing apical ventricular resection (Kikuchi et al., 2010; Porrello et al., 2011); and (4) implantation of unseeded matrices. However, the application of these strategies is still limited since both providing cells with the fundamental signaling without resorting to structural supports and inducing cellular migration into unseeded implanted matrices are challenging issues (Bilodeau and Mantovani, 2006). The complexity of the scenario is augmented by the fact that mammalian hearts have a regenerative potential only for a brief period after birth that is lost during development (Porrello et al., 2011). On the contrary, *in vitro* tissue development was proven to be more effective and adaptive, with its three main components, i.e., cells, scaffolds and culture environment, that can be used individually or in combination (Lyons et al., 2008): (1) cells synthesize the new tissue; (2) scaffolds provide physical support to cells and a structural and biochemical cue tailored to promote cell adhesion, migration, proliferation and differentiation (e.g., allowing the application of physical stimuli on the engineered construct); (3) biomimetic *in vitro* culture environments, designed to replicate the *in vivo* milieu by using biologically inspired requirements, influence and drive cells to differentiate towards the desired phenotype and to express their functions, promoting extracellular matrix (ECM) formation and tissue maturation (Grayson et al., 2009; Egli and Luginbuehl, 2012).

Due to the structural and functional complexity of cardiac tissues, for the effective production of organized and functional cardiac engineered constructs *in vitro*, two fundamental requirements need to be satisfied. Firstly, cardiac TE implies the use of biomaterials that might present native-like tissue mechanical properties and/or topographical cues (Kim et al., 2010), as well as applying physiologic conditions such as perfusion (Radisic et al., 2008), electrical (Tandon et al., 2009a) and mechanical stimulation (Fink et al., 2000; Zimmermann et al., 2002a; Gonen-Wadmany et al., 2004; Birla et al., 2007) during cell culture (Chiu and Radisic, 2013, Massai et al., 2013). Consequently, a suitable dynamic environment is essential for applying these physiological conditions, and can be achieved and maintained within bioreactors, technological devices that, while monitoring

and controlling the culture environment and stimulating the construct, attempt to mimic the physiological milieu (Massai et al., 2013).

It has been demonstrated, in fact, that the possibility (1) to monitor and control the physicochemical environment, (2) to provide a wide range of physical stimuli, and, eventually, (3) to adapt culture conditions to tissue maturation through the use of bioreactor-based systems, allows to obtain engineered constructs with improved morphological and functional properties (Carrier et al., 1999; Carrier, 2000; Dumont et al., 2002; Korossis et al., 2005; Bilodeau and Mantovani, 2006). Moreover, in recent years, several studies have shown that the use of bioreactors in industrial processes for TE is sustainable both clinically and economically (Archer and Williams, 2005; Pörtner et al., 2005; Martin et al., 2009; Olmer et al., 2012), demonstrating that the use of closed, standardized, and automated systems guarantees more reproducibility and lower contamination risk than production processes carried out manually (Ratcliffe and Niklason, 2002; Shachar et al., 2003; Sen et al., 2004; Bouten et al., 2011)[1].

Moreover, cardiac TE has shown the potential to generate thick-contractile myocardium-like constructs that might be used as functional substitutes or as biological *in vitro* model systems (Bursac et al., 1999; Habeler et al, 2009; Smits et al., 2009; Hirt et al., 2012) to investigate the cardiac tissue-specific development and diseases, and to offer accurate and controlled *in vitro* tests for cell and cardiac tissue-based therapies (Tulloch and Murry, 2013), drug screening, predictive toxicology and target validation (Elliot and Yuan, 2010; Hansen et al., 2010).

Within this scenario and starting from these premises, the objectives of this work concern two fundamental aspects of the cardiac TE strategies, in particular the design and the development of dedicated bioreactors, and the generation of a thick-relevant contractile cardiac patch by using a bioreactor-based culture system.

As already discussed, the use of bioreactors allows to obtain several advantages in the attempt to mimic the physiological milieu (Bilodeau and Mantovani, 2006; Massai et al., 2013). Within this context, the developed bioreactor prototypes represent innovative support devices for cardiac TE that can be used with a dual purpose. Firstly, they can be used as model systems to investigate *in vitro* the effects of mass transport and biophysical and chemical stimuli on cardiac cell culture and cardiac tissue formation and maturation, and secondly, once optimal culture conditions have been identified, bioreactors can be used as production systems for *in vitro* generation of engineered functional cardiac tissues.

Similarly, literature data (Pok et al., 2011; Venugopal et al., 2012) report the beneficial effects of the use of patches for the injured myocardium. Within this scenario, our study provide proof-of-principle for generating a thick-relevant cardiac SM-based contractile 3D

[1] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

patch to be used as functional substitutes or as biological *in vitro* research model systems for assessing fundamental myocardial biology and physiology, and for use in pharmacological research.

The clinical motivation of this work comes from the potential clinical significance of combining these two complementary aspects of cardiac TE: in the near future, to develop innovative technological and biological tools to be used as model systems could strongly support the investigation of the still unknown mechanisms of cardiac tissue development, and in the late future, such approach could represent an effective therapeutic strategy to the reestablishment of the structure and function of injured cardiac tissues in the clinical practice, potentially contributing to the improvement of quality of life.

## 2. Anatomy, properties and pathologies of the human heart

### *2.1 The heart*

The mammalian heart is a muscular pump which serves two primary functions: (1) collect blood from the tissues of the body and pump it to the lungs; and (2) collect blood from the lungs and pump it to all other tissues in the body. The human heart lies in the protective thorax, posterior to the sternum and costal cartilages, and rests on the superior surface of the diaphragm. The heart assumes an oblique position in the thorax, with two-thirds to the left of midline. It occupies a space between the pleural cavities called the middle mediastinum, defined as the space inside of the pericardium, the covering around the heart (Figure I.1). This serous membrane has an inner and an outer layer, with a lubricating fluid in between. The fluid allows the inner visceral pericardium to "glide" against the outer parietal pericardium (Weinhaus and Roberts, 2009).

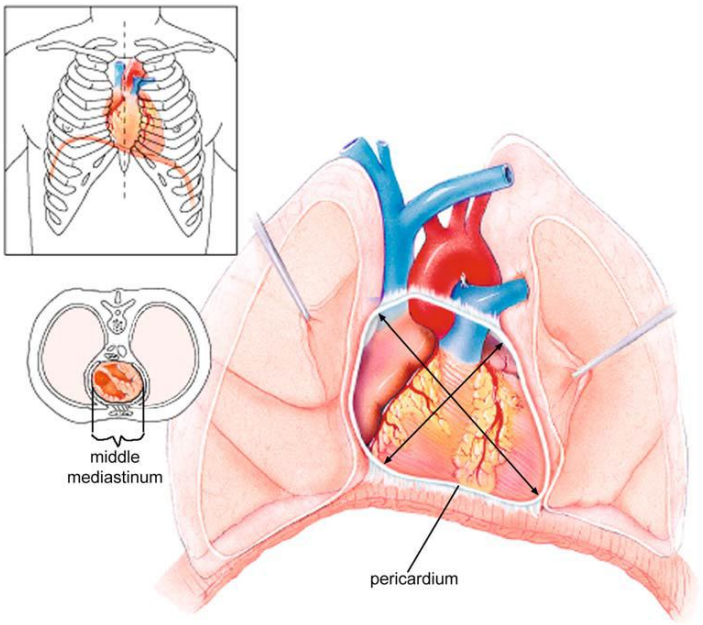


**Figure I.1.** Position of the heart in the thorax. (Weinhaus and Roberts, 2009).

The internal anatomy of the heart reveals four chambers composed of cardiac muscle or "myocardium". The two upper chambers (or atria) function mainly as collecting chambers; the two lower chambers (ventricles) are much stronger and function to pump blood. The role of the right atrium and ventricle is to collect blood from the body and pump it to the lungs. The role of the left atrium and ventricle is to collect blood from the lungs and pump it throughout the body. There is a one-way flow of blood through the heart; this flow is maintained by a set of four valves. The atrioventricular or AV valves (tricuspid and bicuspid) allow blood to flow only from atria to ventricles. The semilunar valves (pulmonary and aortic) allow blood to flow only from the ventricles out of the heart and through the great arteries (Figure I.2) (Weinhaus and Roberts, 2009).

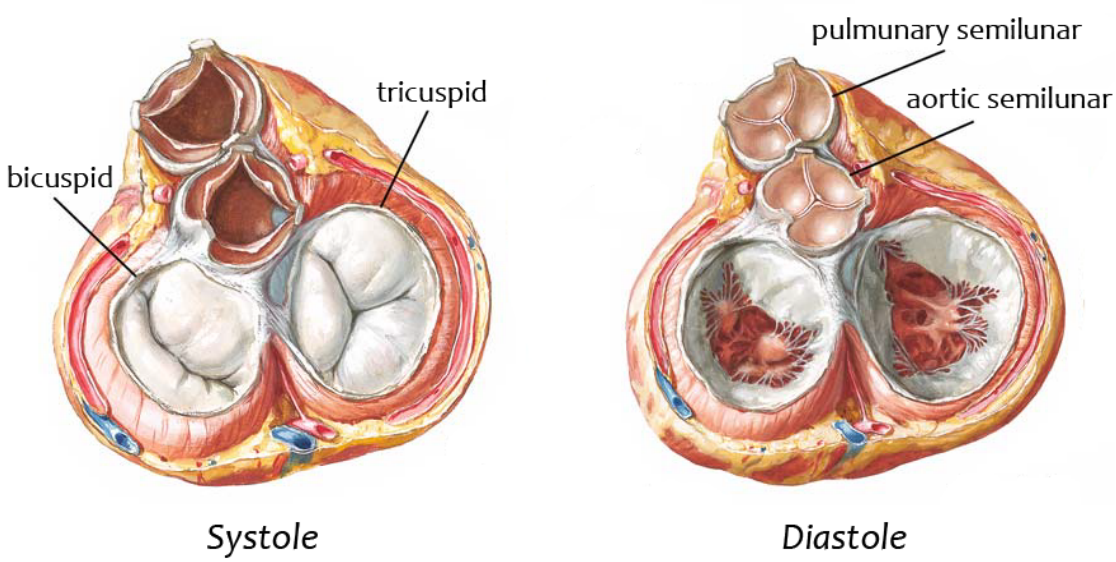


**Figure I.2.** Valves of the heart. The right AV valve is the tricuspid valve, the left is the bicuspid valve. The valve of the pulmonary trunk is the pulmonary semilunar valve, and the aortic artery has the aortic semilunar valve (Weinhaus and Roberts, 2009).

During ventricular systole, AV valves close in order to prevent the regurgitation of blood from the ventricles into the atria. During ventricular diastole, the AV valves open as the ventricles relax, and the semilunar valves close (Figure I.2). The semilunar valves prevent the backflow of blood from the great arteries into the resting ventricles.

Although the heart is filled with blood, it provides very little nourishment and oxygen to the tissues of the heart. The walls of the heart are too thick to be supplied by diffusion alone. Instead, the tissues of the heart are supplied by a separate vascular supply committed only to the heart. The arterial supply to the heart arises from the base of the aorta as the right and left coronary arteries (running in the coronary sulcus). The venous drainage is via cardiac veins that return deoxygenated blood to the right atrium. The coronary arteries arise from the ostia in the left and right sinuses of the aortic semilunar valve, course within the epicardium, and encircle the heart in the AV (coronary) and interventricular sulci (Figure I.3) (Weinhaus and Roberts, 2009).

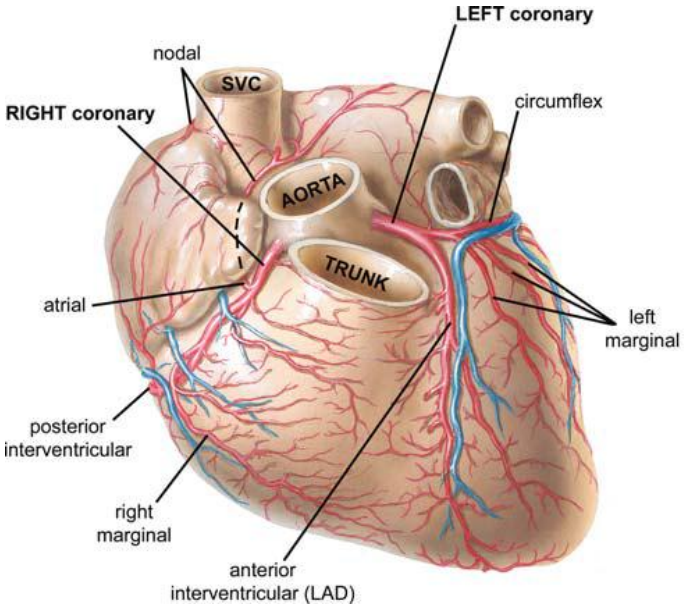


**Figure I.3.** Vascular supply to the heart. Arterial supply to the heart occurs via the right and left coronary arteries and their branches. Venous drainage occurs via cardiac veins (Weinhaus and Roberts, 2009).

A cross-section cut through the heart reveals a number of layers (Figure I.4). From superficial to deep these are: (1) the parietal pericardium with its dense fibrous layer, the fibrous pericardium; (2) the pericardial cavity (containing only serous fluid); (3) a superficial visceral pericardium or epicardium (epi = "upon" + "heart"); (4) a middle myocardium (myo="muscle"+"heart"); and (5) a deep lining called the endocardium (endo = "within"). The endocardium is the internal lining of the atrial and ventricular chambers, and is continuous with the endothelium (lining) of the incoming veins and outgoing arteries. It also covers the surfaces of the AV valves, pulmonary and aortic valves, as well as the chordae tendinae and papillary muscles. The endocardium is a sheet of epithelium called endothelium that rests on a dense connective tissue layer consisting of elastic and collagen fibers. These fibers also extend into the core of the previously mentioned valves (Weinhaus and Roberts, 2009).

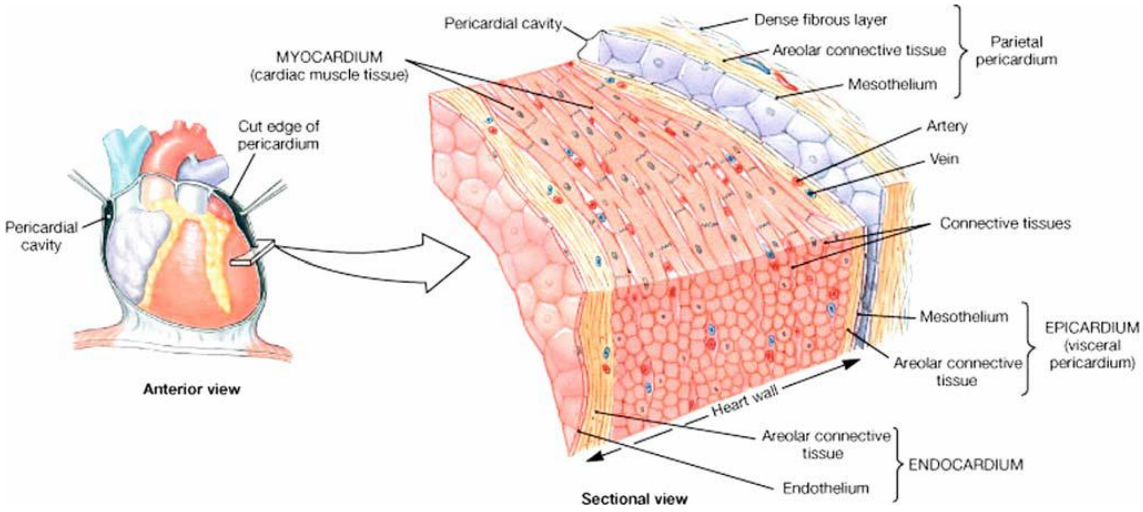


**Figure I.4.** Internal anatomy of the heart. The walls of the heart contain three layers—the superficial epicardium, the middle myocardium composed of cardiac muscle, and the inner endocardium (Weinhaus and Roberts, 2009).

The bulk of the heart tissue is the contractile myocardium, a highly differentiated tissue, ~1 cm thick in humans, with an asymmetrical and helical architecture (Buckberg, 2002; Akhyari et al., 2008), composed of tightly packed CMs (forming myofibers), fibroblasts (FBs), endothelial cells (ECs) and smooth muscle cells (SMCs). CMs are highly metabolically active; therefore oxygen and nutrients are depleted within a relatively thin layer of viable tissue (Carrier et al., 2002b). They comprise only 20–40% of the total cells in the heart but they occupy 80–90% of the heart volume (Nag, 1980). Morphologically, intact CMs have an elongated, rod shaped appearance. Contractile apparatus of CMs consists of sarcomeres arranged in parallel myofibrils (Severs, 2000). The cells are supported by a dense vasculature and an ECM that is rich in collagen and laminin. Electrical signals propagate through a 3D syncytium formed by CMs. Rapid impulse propagation is enabled by specialized junctions between cells, gap junctions, which are composed of different forms of connexin protein. The most abundant protein in ventricular CMs is connexin-43. Groups of specialized CMs (pace makers), fastest of which are located in the sinoatrial node; drive periodic contractions of the heart. Majority of the CMs in the myocardium are non-pace maker cells and they respond to the electrical stimuli generated by pace maker cells. Excitation of each CM causes an increase in the amount of cytoplasmic calcium, which triggers mechanical contraction. The result is an electrical excitation leading to a coordinated mechanical contraction to pump the blood forward (Odedra et al., 2011).

Therefore, the heart can be considered as a dynamic electromechanical system where the myocardial tissue undergoes mechanical stretch during diastole and active contraction during systole, consuming large amounts of oxygen.

The physiological stimuli that affect the entire cardiac system submit it to continuous stresses that require an enormous strength, flexibility and durability of the

structures, as well as a high degree of adaptive capacity to cope with changes due to growth, physical activity and pathological conditions (Bouten et al., 2011). In details, in the normal human heart during one cardiac cycle, the left ventricular pressure ranges between 10 and 120 mmHg, and the cavity volume varies between 40 and 130 ml (Fortuin et al., 1971; Bouten et al., 2011), respectively. The right ventricle pressure ranges between 5 to 30 mmHg (Schneck et al., 2003), and the cavity volume changes from 24 to 86.5 ml (Mahoney et al., 1987). Local mechanical loads can reach 50 kPa (Bouten et al., 2011), with 22.9% longitudinal and 59.2% radial mean strain (Kuznetsova et al., 2008). Active contraction forces of myocardial strips isolated from native human ventricles were found to range between 14.5 ± 4.4 and 22.8 ± 1.4 $mN/mm^2$ for healthy donors (Mulieri et al., 1992; Holubarsch et al., 1998). End diastolic values for the Young's modulus have been reported to range between 0.2 and 0.5 MPa (Jawad et al., 2007); however, exhaustive quantitative measures of the mechanical properties of human heart are still an open challenge.

With regard to the electrical properties, tissue in general is surrounded by extracellular fluid with relatively high electrical conductivity (3-12 mS/cm) (Durand, 2000). For vertebrates, the physiologically significant range of endogenously produced electrical field strengths is 0.1-10 V/cm (Tandon et al., 2009a). The electrical stimuli present in the heart can be classified as: (1) direct current (DC) signals, which affect and direct cell migration during the development of the cardiac primitive streak and left-right asymmetry; and (2) the pulsatile signals implicated in the development of the cardiac syncytium (Nuccitelli, 1992). In terms of frequency, the typical resting heart rate in adults is 60-100 beats per minute (bpm) that corresponds to 1-1.7 Hz (Schneck, 2003; Klingensmith et al., 2008). In terms of pulse duration, 1-2 ms is sufficiently long to excite heart tissue cells (Tandon et al., 2009a).

A summary of the heart properties and stimuli is reported in Table I.1**.**

**Table I.1.** Properties and stimuli of human heart[2].

| Property/Stimulus | Heart |
|---|---|
| **Internal diameter** | Left ventricle: 3.3-7.5 cm (Devereux and Reichek, 1977) |
| **Wall thickness** | Left ventricle: 8-15 mm (Bouten et al., 2011)<br>Right ventricle: 4-5 mm (Bouten et al., 2011) |
| **Length** | - |
| **Volume** | Left ventricle: 40-130 ml (Bouten et al., 2011; Fortuin et al., 1971)<br>Right ventricle: 24-86.5 ml (Mahoney et al., 1987) |
| **Resting rate** | 60-100 bpm (1-1.7 Hz) (Schneck, 2003; Klingensmith et al., 2008) |
| **Young's Modulus** | 0.2-0.5 MPa (end diastolic value) (Jawad et al., 2007) |
| **Pressure** | Left ventricle: 10-120 mmHg (Bouten et al., 2011)<br>Right ventricle: 5-30 mmHg (Schneck, 2003) |
| **Mechanical load** | 50 kPa (Bouten et al., 2011) |
| **Strain** | Longitudinal: 22.9% (Kuznetsova et al., 2008)<br>Radial: 59.2% (Kuznetsova et al., 2008) |
| **Electrical field** | 0.1-10 V/cm (Tandon et al., 2009a) |
| **Electrical pulse duration** | 1-2 ms (Tandon et al., 2009a) |

bpm: beats per minute

### 2.2 *The myocardial infarction*

Acute MI remains a leading cause of morbidity and mortality worldwide. MI occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis. Ischemia at this critical threshold level for an extended period results in irreversible myocardial cell damage or death (Bolooki and Askari, 2010).

Most MIs are caused by a disruption in the vascular endothelium associated with an unstable atherosclerotic plaque that stimulates the formation of an intracoronary thrombus, which results in coronary artery blood flow occlusion. If such an occlusion persists for more than 20 minutes, irreversible myocardial cell damage and cell death will occur (Bolooki and Askari, 2010) (Figure I.5).

[2] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

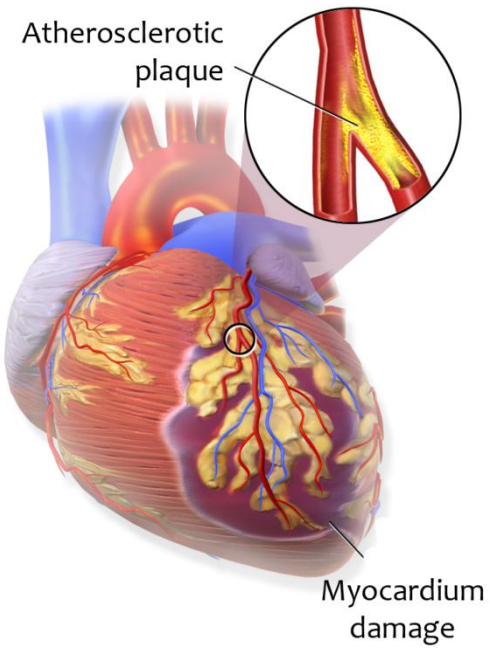


**Figure I.5.** Schematic of the MI. A MI occurs when an atherosclerotic plaque slowly builds up in the inner lining of a coronary artery and then suddenly ruptures, causing catastrophic thrombus formation, totally occluding the artery and preventing blood flow downstream
(Adapted from http://en.wikipedia.org/wiki/Myocardial_infarction).

The development of atherosclerotic plaque occurs over a period of years to decades. The two primary characteristics of the clinically symptomatic atherosclerotic plaque are a fibromuscular cap and an underlying lipid-rich core. Plaque erosion can occur because of the actions of matrix metalloproteases and the release of other collagenases and proteases in the plaque, which result in thinning of the overlying fibromuscular cap. The action of proteases, in addition to hemodynamic forces applied to the arterial segment, can lead to a disruption of the endothelium and fissuring or rupture of the fibromuscular cap. The loss of structural stability of a plaque often occurs at the juncture of the fibromuscular cap and the vessel wall, a site otherwise known as the shoulder region. Disruption of the endothelial surface can cause the formation of thrombus via platelet-mediated activation of the coagulation cascade. If a thrombus is large enough to occlude coronary blood flow, a MI can result (Bolooki and Askari, 2010).

The death of myocardial cells first occurs in the area of myocardium most distal to the arterial blood supply: the endocardium. As the duration of the occlusion increases, the area of myocardial cell death enlarges, extending from the endocardium to the myocardium and ultimately to the epicardium. The area of myocardial cell death then spreads laterally to areas of watershed or collateral perfusion. Generally, after a 6- to 8-hour period of coronary occlusion, most of the distal myocardium has died. The extent of myocardial cell death defines the magnitude of the MI. If blood flow can be restored to at-risk myocardium, more heart muscle can be saved from irreversible damage or death (Bolooki and Askari, 2010).

MI can be subcategorized on the basis of anatomic, morphologic, and diagnostic clinical information. From an anatomic or morphologic standpoint, the two types of MI are transmural and non-transmural. A transmural MI is characterized by ischemic necrosis of the full thickness of the affected muscle segment(s), extending from the endocardium through the myocardium to the epicardium. A non-transmural MI is defined as an area of ischemic necrosis that does not extend through the full thickness of myocardial wall segment(s). In a non-transmural MI, the area of ischemic necrosis is limited to the endocardium or to the endocardium and myocardium. It is the endocardial and subendocardial zones of the myocardial wall segment that are the least perfused regions of the heart and the most vulnerable to conditions of ischemia (Bolooki and Askari, 2010).

The severity of a MI depends on three factors: the level of the occlusion in the coronary artery, the length of time of the occlusion, and the presence or absence of collateral circulation. Generally, the more proximal the coronary occlusion, the more extensive the amount of myocardium that will be at risk of necrosis. The larger the MI, the greater the chance of death because of a mechanical complication or pump failure. The longer the period of vessel occlusion, the greater the chances of irreversible myocardial damage distal to the occlusion (Bolooki and Askari, 2010). As a consequence, a marked inflammatory response is elicited, developing in parallel to a dramatic decrease in nutrients and oxygen supply, and dead cells are removed by macrophages. Over the subsequent weeks to months, FBs and ECs migrate and form granulation tissue that ultimately becomes a thick and stiff collagenous scar. Scar formation reduces contractile function of the heart, heavily affects mechanical and electrical properties of native heart muscle, and leads to ventricle wall remodeling, and ultimately to heart failure (Vunjak-Novakovic et al., 2010).

Currently, there are no methods that can completely prevent these pathological events, however research is currently under way on the approaches that can stabilize the scar faster, reduce the expansion of the infarct, and decrease ventricular wall stresses that lead to further remodeling events. Treatments that limit the remodeling that occurs post infarction will improve the survival and quality of life of MI patients (Pfeffer et al., 1990).

## 3. Cardiac tissue engineering

Currently, there are a variety of methods to attenuate the effects of MI, many of which are still in the preclinical stage. Among different methods, cardiac TE is become a promising strategy that offers the possibility of creating functional tissue equivalents for scientific studies and tissue repair. The motivation for engineering cardiac muscle is two-fold: 1) a 3D culturing system may results in an easily accessible accurate *in vitro* model of natural cardiac tissue for use in physiological and pharmacological research, and 2) engineered tissue may eventually be used for the repair of damaged heart tissue. Therefore, as pointed

out in this thesis, the role of bioreactors (Chapter II and III) and scaffolds (Chapter IV), in the form of cardiac patches, is fundamental for developing cardiac TE strategies.

The following sections provide an up-to-date review of the current developed bioreactors and cardiac patches. Firstly, bioreactors for *in vitro* investigation and generation of cardiac tissues are illustrated. Afterwards, cardiac patches as biological *in vitro* model systems to investigate the cardiac tissue-specific development and diseases or as functional cardiac substitutes are described.

### *3.1 Bioreactors*[3]

Generation of tissue with complex structure and function is not feasible by culturing cells within Petri dish systems, since without the appropriate chemico-physical stimuli and 3D environment, cells cannot maintain their shapes, phenotypes, and roles, and lose the ability to proliferate and form organized tissues (Bilodeau and Mantovani, 2006). Many attempts have been made to culture cells in environments mimicking, at least partially, the *in vivo* milieu, where mechanical load, electrical stimuli, perfusion pressure, autocrine/paracrine and systemic hormonal stimulation are necessarily interwoven (Fink et al., 2000). The use of bioreactors properly designed to build up *in vitro* cell culture models allowed to study the effects of biophysical factors under closely monitored and tightly controlled culture conditions and to generate tissues *in vitro* (Martin et al., 2004; Bilodeau and Mantovani, 2006; Wendt et al., 2009). Bioreactors are closed, standardized and almost operator-independent systems assuring greater reproducibility, traceability, scalability, and lower contamination risk than traditional manual processes. Furthermore, bioreactors can be easily optimized according to present and future regulations (Ratcliffe and Niklason, 2002; Sen et al., 2004).

The use of bioreactors allows to make automated, repeatable, scalable and clinically sustainable biological processes such as: (1) cell expansion; (2) cell seeding of scaffolds; (3) cellular differentiation and tissue maturation; (4) effects of drugs on cells and engineered tissues (drug screening); and (5) *in vitro* disease model investigation. Moreover, they can be used as model systems for the investigation of cell functions and tissue development in specific environmental conditions (concentrations of oxygen, carbon dioxide, nutrients and biochemical factors; hydrodynamic conditions; physical stimuli) (Freed et al., 2006; Bouten et al., 2011). Advanced bioreactor systems should be equipped with the following (Figure I.6): (a) sensors and control systems for a real time, automatic monitoring and control of culture parameters (i.e., temperature, pH, biochemical gradients, gas concentrations, pressure, mechanical and electrical stresses, waste removal, etc.) within the culture

[3] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

chamber; (b) recirculation/perfusion systems assuring medium replacement and optimized cell and nutrient distribution within the 3D environment; and (c) physical stimulation systems for mimicking the native physiological conditions and supporting tissue maturation (Bilodeau and Mantovani, 2006; Martin et al., 2009). Therefore, for an effective *in vitro* tissue generation strategy, it is essential to define not only culture actors (cells, scaffolds, culture medium, growth factors, etc.), but also bioreactor's design criteria, which are dictated by the tissue to be produced (Barron et al., 2003; Bilodeau and Mantovani, 2006).

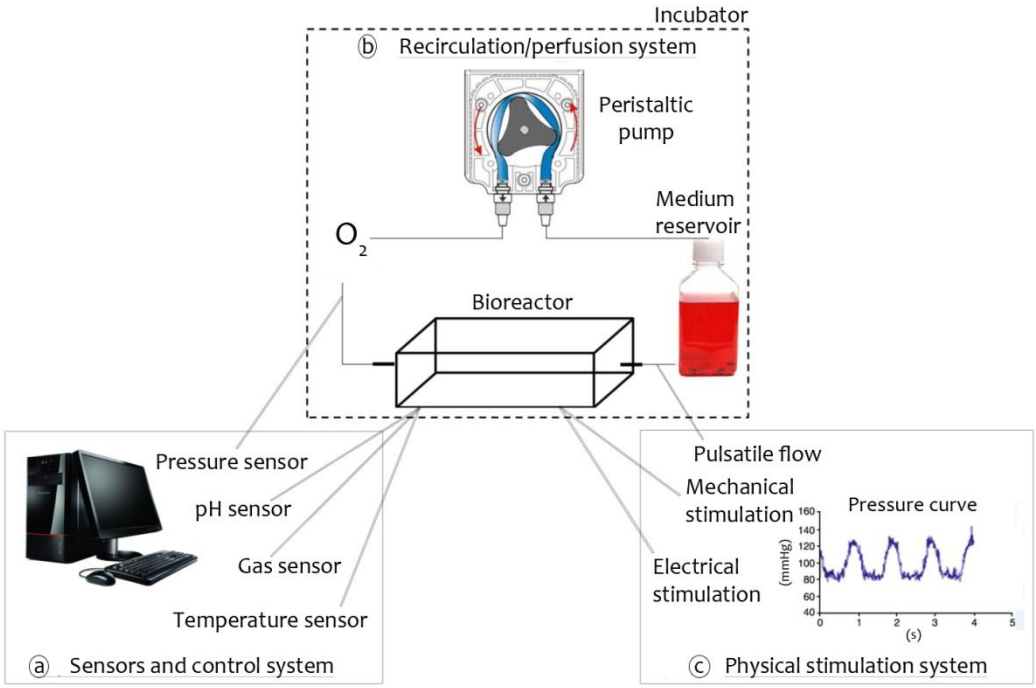


**Figure I.6.** Advanced bioreactor system equipped with (a) sensors and control systems, (b) recirculation/perfusion systems, and (c) physical stimulation systems.

Focusing on bioreactors designed for the production of cardiac constructs suitable for implantation, the presence of physical stimulation systems (i.e., mechanical stretching, electrical stimulation) is fundamental for promoting the modulation of cell behavior in terms of proliferation (Nasseri et al., 2003), differentiation (Tandon et al., 2008), protein synthesis and ECM remodeling (Mertshing and Hansmann, 2009), and for promoting structural and functional tissue maturation in terms of 3D morphology (Carrier et al., 1999), mechanical strength (Gonen-Wadmany et al., 2004) and electrical function (Tandon et al., 2009a). Hence, the complexity of the *in vivo* environment requires the fulfillment of design criteria assuring proper stimulation in terms of pulsatile forces characterized by pressures and frequency, shear stresses at physiologic frequencies (i.e., heart rate) and physiologic flow rate, stroke volume, and stroke rate values (Lyons and Pandit, 2005). An overview of the state of the art of bioreactors is provided below for the study of phenomena involved in

the mechanism of cardiac tissue formation, and for the *in vitro* generation of cardiac tissues.

### *3.1.1 Bioreactors for cardiac tissue engineering*[4]

As already discussed, the human heart has a limited capacity to regenerate itself (Papadaki et al., 2001). Therefore, the generation of 3D engineered cardiac patches to be implanted into the injured myocardium represents a challenging but effective and promising therapeutic strategy. However, cardiac TE is still in the research phase, because several problems are still to be solved to achieve full recovery of a damaged region. In detail, an engineered cardiac patch should (1) have dimensions (typically 10-50 $cm^2$ of surface area and several millimeters of thickness) and contractile features adequate to support failing hearts, thus vascularization is fundamental for its survival; (2) have a compliant response adequate to assure adaptation to systolic strength and diastolic relaxation; and (3) guarantee structural and electrical integration with the hosting myocardium (Zimmermann et al., 2006; Vunjak-Novakovic et al., 2010). The complexity of the cardiac tissue makes the fulfillment of these requirements very challenging, since adult CMs quickly dedifferentiate *in vitro* and the maintenance of their differentiation *in vitro* is still an open issue, and neonatal cells are still immature to obtain effective results from their culture. These open issues have driven the development of the biomimetic paradigm of cardiac TE, which involves the application of physiologically-relevant chemical and physical stimuli to cultured cells (Tandon et al., 2009a). Table I.2 summarizes a selection of studies, described in detail and ordered with respect to the provided physical stimuli and flow conditions, where it is demonstrated that a synergistic combination of cells, scaffolds and culture conditions within tailored bioreactors allows to obtain cardiac engineered constructs which are close to the native tissue in morphology and function, thus offering new perspectives to basic cardiac research and tissue replacement therapy.

[4] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

**Table I.2.** Devices and bioreactors for cardiac tissue engineering.

| Authors | Cells | Scaffold | Device | Physical Stimulation | Monitoring | Contraction forces |
|---|---|---|---|---|---|---|
| **Eschenhagen** *et al*., **1997** | Embryonic chick CMs | Cell/type I collagen mixture | Organ bath (static flow) | Stepwise adjusted preloads<br><br>Electrical rectangular pulse (10 ms, 20-40 V, 0.8-2 Hz) | Contractile activity | Resting tension: 1.15-2.81 mN<br><br>Twitch tension: 0.09-0.2 mN<br><br>Beating: 72.4 ± 2.6 bpm |
| **Zimmermann** *et al*., **2000** | Neonatal rat CMs | Cell/type I collagen/Matrigel mixture | Organ bath (static flow) | Stepwise adjusted preloads<br><br>Electrical rectangular pulse (5 ms, 1 Hz) | Contractile activity | Resting tension: 0.20-0.63 mN<br><br>Twitch tension: 0.21-0.46 mN<br><br>Beating: 180 bpm |
| **Fink** *et al*., **2000** | Embryonic chick CMs<br><br>Neonatal rat CMs | Cell/type I collagen/Matrigel (rat) mixture | Stretch device (static flow)<br>Organ bath (static flow) | Cyclic unidirectional stretch (1-20%, 1.5 Hz)<br><br>Stepwise adjusted preloads | Contractile activity | Force of contraction of stretched (20%) ETHs: 0.10-0.18 mN (chicken, 238% over controls) |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Electrical rectangular pulse (10 ms, 20-40 V, 1.5 Hz (chick) or 2 Hz (rat)) | | 0.20-0.65 mN (rat, 188% over controls)<br><br>Twitch duration: decrease of 14-44% with respect to unstretched controls |
| **Zimmermann** *et al*., **2002a** | Neonatal rat CMs | Cell/type I collagen/Matrigel mixture | Stretch device (static flow) | Cyclic unidirectional stretch (10%, 2 Hz) | Contractile activity | Resting tension: 0.05-0.27 (basal condition) mN |
| | | | Organ bath (static flow) | Stepwise adjusted preloads electrical rectangular pulse (1Hz) | | Twitch tension: 0.36 (basal condition) -0.75 mN |
| **Gonen-Wadmany** *et al*., **2004** | Neonatal rat CMs and sheep aortic SMCs | Type I collagen enriched with growth factors and hormones | Mechanical stimulation bioreactor (static flow) | Cyclic strain (0-12% cyclic change in the outer diameter of each bulb, 1 Hz) | - | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| **Birla** *et al*., **2007** | Neonatal rat CMs | Fibrin gel with thrombin | Mechanical stimulation bioreactor (static flow) | Cyclic stretch (10%, 1 Hz)<br><br>Parallel electrodes (10 ms, 15 V, 1 Hz) with custom-build optical force transducer | Contractile activity | Mean specific active force: 12.1 ± 0.62 $kN/m^2$ |
| **Akins** *et al*., **1999** | Neonatal rat CMs | Fibronectin-coated polystyrene beads<br><br>Oriented collagen fibers | HARV rotating bioreactor (laminar flow)<br><br>Transparent teflon culture bags (slowly rotated) | - | Contractile frequency (real time for Teflon culture bags) | - |
| **Carrier** *et al*., **1999** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous PGA | Flsks (static and stirred turbulent flow, 50-90 rpm)<br><br>Dishes mounted on *xyz* gyrator (mixed flow, 24 rpm) | - | Culture parameters (pH, $pO_2$, $pCO_2$)<br><br>Cell damage and death (measuring medium lactate dehydrogenase | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Rotating-wall bioreactor (dynamic laminar flow, 11-12 rpm) | | Levels in supernatant) | |
| **Papadaki** *et al*., **2001** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous laminin-coated sPGA | Flasks (stirred turbulent flow, 50 rpm) dishes mounted on *xyz* gyrator (mixed flow, 25 rpm)<br><br>Rotating-wall bioreactor (dynamic laminar flow, 12 rpm) | Electrical pulses (1 ms, 0.1-5 V at a rate of 60 bpm) | Electro-physiological properties | - |
| **Carrier** *et al*., **2002a; 2002b** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous PGA | Flasks (stirred turbulent flow, 50 rpm)<br><br>Direct perfused cartridges (1h at 0.2 ml/min, and then 10 days at 0.6, 1 or 3 ml/min)<br><br>Electrophysiological assessment | - | Culture parameters (pH, $pO_2$, $pCO_2$) | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | apparatus | | | |
| **Radisic** *et al*., **2003** | Neonatal rat CMs (feasibility studies)<br><br>Murine C2C12 cells (detailed seeding studies) | Collagen sponges enriched with Matrigel | Orbitally mixed dishes (25 rpm)<br><br>Direct perfused cartridges (seeding: alternating flow; cultivation in a perfusion loop: unidirectional flow, 0.5 ml/min)<br><br>Electrophysiological assessment system | Electrical square pulses (2 ms, 1-5 V, 1 Hz) | Contractile activity | - |
| **Radisic** *et al*., **2004** | Neonatal rat CMs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (orbitally mixed flow, 25 rpm) | Electrical rectangular pulse (2 ms, 5 V/cm, 1 Hz) | Contractile activity (real time)<br>Electrical activity | - |
| **Tandon** *et al*., **2008; 2009a; 2011** | Neonatal rat CMs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (static flow) | Electrical square monophasic pulses (2 ms, 0-12.5 V/cm, 1 Hz) | Contractile activity (real time) | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| **Tandon** *et al*., **2009b** | Human adipose tissue-derived SCs<br><br>Human epicardial adipose tissue-derived SCs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (static flow) | Electrical DC fields (6 V/cm) | - | - |
| **Barash** *et al*., **2010** | Neonatal rat CMs | Alginate porous scaffolds | Electrical stimulation and perfusion bioreactor (25 ml/min) | Electrical bipolar pulses (2 ms, 5 V, 1 Hz) | Culture parameters (pH, $pO_2$, $pCO_2$) | - |
| **Maidhof** *et al*., **2012** | Neonatal rat cardiac cells | Channeled PGS | Perfused | Electrical square monophasic pulses (3 V/cm, 3 Hz) | Contractile activity | - |
| **Hansen** *et al*., **2010** | Neonatal rat CMs | Cell/fibrinogen/ Matrigel/ thrombin mixture | Minuaturized drug screening platform (static flow) | - | Contractile activity (real time) | Force: 0.05 to 0.4 mN (organ baths: force up to 0.9 mN, relative force up to 28.7 $mN/mm^2$) frequency: 0.3-2.7 Hz |
| **Schaaf** *et al*., **2010** | Human ESC-derived CMs | Cell/fibrinogen/ Matrigel/thrombin mixture | Miniaturized drug screening platform (static flow) | - | Contractile activity (real time) | Force: |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 0.12 mN/mm$^2$<br>Frequency:<br>0.5 Hz |
| **Kensah** *et al*., **2011** | Neonatal rat CMs | Cell/type I collagen/ Matrigel mixture | Mechanical stimulation bioreactor (static flow) | Cyclic longitudinal stretch (10%, 1 Hz) stepwise increased preloads electrical rectangular pulse (5 ms, 25 V) | Contractile activity (real time) Live cell imaging | Maximum spontaneous systolic force:<br>0.96 ± 0.09 mN (controls)<br>1.42 ± 0.09 mN (stretch alone)<br>2.54 ± 0.11 mN (noradrenalin) |
| **Kensah** *et al*., **2013** | Murine pluripotent SC-derived CMs<br><br>Human embryonic and pluripotent SC-derived CMs | Cell/Type I collagen/Matrigel mixture | Mechanical stimulation bioreactor (static flow) | Constant static stretch uniaxial cyclic Stretch (10%, 1 Hz) Growing static stretch (stepwise elongation by 200 µm) | Contractile activity (real time) Live cell imaging | Active force:<br>0.97 ± 0.07 mN (static stretch)<br>0.77 ± 0.07 mN<br><br>(cyclic stretch)<br>1.22 - 1.42 mN (ascorbic acid + growing static stretch)<br><br>Maximum specific active force: |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 4.4 mN/mm$^2$ (human) |
| **Boudou** *et al.*, **2012** | Neonatal rat CMs | Collagen/fibrin 3D micropatterned matrices | MEMS cantilevers | Electrical biphasic square pulses (1 ms, 6 V/cm, 0.2 Hz) | Contractile activity (real time) | - |

PGA: poly-glycolic acid; sPGA: surface-hydrolyzed PGA; PGS: poly-glycerol-sebacate; $pO_2$: partial pressure of oxygen; $pCO_2$: partial pressure of carbon dioxide; rpm: revolution per minute.

In their pioneering studies (Eschenhagen et al., 1997; Zimmermann et al., 2000; Eschenhagen et al., 2002b), Eschenhagen, Zimmermann and coworkers proposed a method for the *in vitro* production of coherently contracting 3D engineered heart tissues (EHTs) made of CMs from embryonic chicken (Eschenhagen et al., 1997) and neonatal rats (Zimmermann et al., 2000; Zimmermann et al., 2002b) mixed with collagen type I and, only when cultivating rat cells, Matrigel. Going beyond the limitations of monolayer cultures, sheet-shaped EHTs provided a simplified model suitable for the investigation of heart-like features of the constructs and for an analysis of the consequences that culture environment and genetic manipulations have on contractility. In order to measure EHT contractile forces, EHTs were then immersed in thermostated organ bathes, and subjected to stable isometric preloads and electrical pulses (more details in Table I.2). EHTs exhibited well organized myofilaments with intercellular connections, and coherent contractions after 2-3 days (Eschenhagen et al., 1997; Zimmermann et al., 2000).

The influence of chronic mechanical stretch on morphological and functional behavior of CMs was evaluated by Fink et al. (Fink et al., 2000), who subjected EHTs to phasic unidirectional stretch (1-20%, 1.5 Hz) for 6 days and then to isometric force measurement (as in (Eschenhagen et al., 1997; Zimmermann et al., 2000)). Stretched EHTs exhibited improved organization of CMs into parallel arrays of rod-shaped cells, increased cell length and width, and a marked improvement of the contractile function.

To overcome the limitation of the inhomogeneous cell distribution affecting sheetshaped EHTs, ring-shaped rat EHTs were cast (Zimmermann et al., 2002a). After 7 days of culture, ring-shaped EHTs were transferred into a modified stretch device (Figure I.7a) and submitted to unidirectional cyclic stretch (10%, 2 Hz) for 7 days; afterward, EHTs were subjected to isometric force measurements (as in (Eschenhagen et al., 1997; Zimmermann et al., 2000)). On circular EHTs, Zimmermann et al. (Zimmermann et al., 2002a) observed complexes of multicellular aggregates and longitudinally oriented cell bundles, with morphological features of adult tissue.

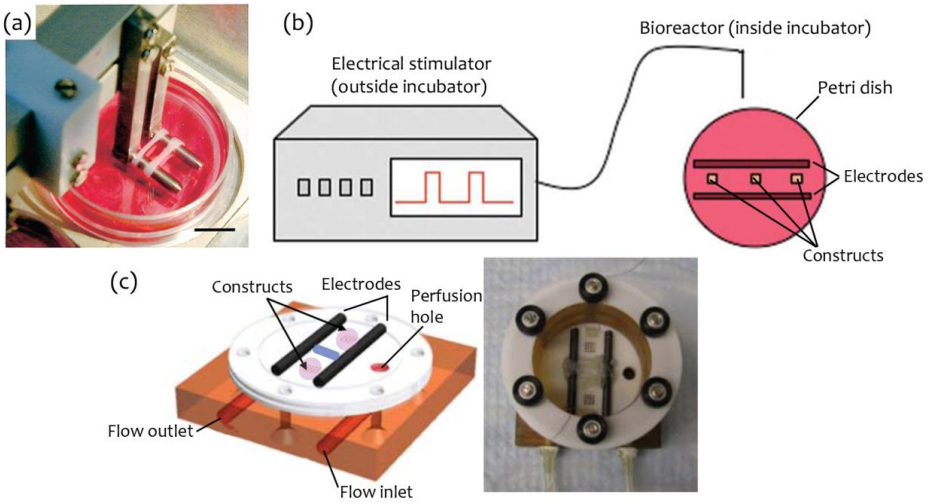


**Figure I.7.** (a) Stretch device developed by Eschenhagen (Eschemhagen et al., 2002b). (b) Bioreactor for electrical stimulation developed by Tandon (Tandon et al., 2009a). (c) Perfusion bioreactor developed by Maidhof (Maidhof et al., 2012).

Eschenhagen, Zimmermann and coworkers (Eschenhagen et al., 2002a; 2002b; Zimmermann et al., 2002b) proved the *in vivo* feasibility of the EHT implantation on rats. Implanted EHTs maintained a network of differentiated CMs and were strongly vascularized (Eschenhagen et al., 2002a; 2002b; Zimmermann et al., 2002b). Moreover, EHTs grafted on the heart of syngenic rats (Zimmermann et al., 2002b) preserved contractile function *in vivo*. More recently, Zimmermann et al. (Zimmermann et al., 2000) evaluated the performance of multiloop EHTs implanted in Wistar rat heart muscles after MI. Large force-generating EHTs were produced under elevated oxygen concentration, under auxotonic load (simultaneous changes in stress and length), and with supplementation of culture medium with insulin. To facilitate implantation, five single circular EHTs were stacked crosswise on a tailored holding device, promoting EHT fusion and allowing contractions under auxotonic load. Synchronously contracting multiloop EHTs were obtained (about 15 mm of diameter and 1–4 mm of thickness), suitable for *in vivo* engraftment. EHTs integrated well and coupled electrically to the hosting myocardium, exerting beneficial effects on systolic and diastolic left ventricular function with normalization of epicardial impulse propagation after engraftment and no evidence of arrhythmogenicity. However, proarrhythmic risks from EHT implantation are likely to be not negligible in larger, low-heart-rate, bigger species including humans.

By adopting a multi-chamber bioreactor to impart controlled simultaneous cyclic strains, Gonen-Wadmany et al. (Gonen-Wadmany et al., 2004) developed a bioartificial engineered cardiac construct (ECC) capable of synchronized multidirectional contraction. Based on previous studies (Zimmermann et al., 2000; 2002a), ECCs were prepared mixing neonatal rat CMs and sheep aortic SMCs with type I collagen gel, enriched with growth factors and hormones. ECCs, molded over silicone bulbs, were mechanically stimulated by

inflating and deflating the silicone bulbs with repetitive pneumatic pressure at 1 Hz for 6 days. Cell distribution was found to be homogeneous throughout the ECCs, and the use of SMCs resulted in a significant compaction of the collagen gels and in a cardiac substitute containing a high cell density. Moreover, the authors demonstrated that cellular and morphological reorganization is highly dependent on the amplitude of strain stimulation.

In 2007, Birla et al. (Birla et al., 2007) proposed a multi-chamber bioreactor capable of controlling tissue stimulation in order to correlate the degree of mechanical stretch to changes in the contractile performance of 3D bioengineered heart muscle (BEHM) constructs. Neonatal rat CMs were plated on fibrin-coated surfaces of cell culture plates, with the addition of thrombin, and maintained in incubator for 2 weeks. Spontaneous contractions of the primary CMs resulted in compaction of the fibrin gel promoting BEHM formation. For another 7 days, BEHMs were mechanically stimulated within the bioreactor with different time intervals per day (2, 6, and 24 hours). The active force was evaluated by stimulating the BEHMs between parallel platinum electrodes (more details in Table I.2). No apparent physical damage was found in BEHMs stretched using a stretch protocol of 10% stretch and 1 Hz, with no significant changes in the active force, specific force, pacing characteristics, or morphological features, demonstrating the structural stability of the constructs in response to applied stretch protocol.

By performing 3D suspension cultures of neonatal rat CMs on cell supports within rotating bioreactor (HARV, Synthecon), Akins et al. (Akins et al., 1999) investigated the capacity of isolated heart cells to re-establish tissue architectures *in vitro*. They observed the formation of 3D aggregates of mixed populations of ventricular cells, replicating the distribution observed *in vivo*, and presenting spontaneous and rhythmic contraction, suggesting that cardiac cells possess an innate capacity to re-establish complex 3D cardiac organization *in vitro*.

Motivated by the need to optimize seeding and perfusion of 3D scaffolds, Carrier et al. (Carrier et al., 1999) used different bioreactors (flasks, xyz gyrator, and rotating bioreactors) to investigate the *in vitro* morphogenesis of engineered cardiac muscle in a cell-polymer bioreactor model system. Porous nonwoven meshes of fibrous PGA were seeded with rat heart cells. Constructs cultivated within rotating bioreactors showed significantly improved structural and functional properties, with uniformly distributed cellularity, improved maintenance of metabolic parameters, elongated cell shape, and ultrastructural features peculiar of native cardiac tissue.

Papadaki et al. (Papadaki et al., 2001) cultivated highly concentrated neonatal rat CMs, seeded on laminin-coated PGA scaffolds, within rotating bioreactors. By using a specific apparatus (Bursac et al., 1999), they stimulated (0.1-5 V, 1 ms, pulses at a rate of 60 bpm) and recorded extracellular potentials. The engineered cardiac muscle presented a peripheral region containing CMs electrically connected through functional gap junctions. These constructs did not exhibit spontaneous beating, but responded to electrical

stimulation and showed conduction velocity of propagating electrical impulses comparable with native tissue.

However, due to the concentration gradients associated with diffusional transport of nutrients and oxygen, the engineered tissues mentioned above were limited to approximately 100-μm-thick peripheral layer around a relatively cell-free construct interior. To overcome this limitation, Carrier et al. investigated the effects of direct perfusion (Carrier et al., 2002b) and oxygen concentration (Carrier et al., 2002a) on engineered cardiac tissues obtained from fibrous PGA scaffolds seeded with neonatal rat CMs. It was found that direct medium perfusion through the constructs, located within cartridges, guarantees the following: (1) a reduction of diffusional gradients over macroscopic distances; (2) the control of local levels of pH and oxygen; (3) the spatial uniformity of cell distribution; and (4) an increase of construct thickness (Carrier et al., 2002b). It was also proven that a marked positive correlation exists between medium pO2 and the aerobicity of cell metabolism, DNA and protein content, and the expression of cardiac-specific markers (Carrier et al., 2002a). However, the system in (Carrier et al., 2002a) had two important limitations: (1) direct perfusion exposes cardiac cells to hydrodynamic shear stress values (0.2-1 dyn/cm$^2$ for perfusion rate 0.6-3 ml/min, respectively) higher than the physiological ones; (2) cell density of engineered tissue was approximately only the 20-25% of the density in native cardiac tissue (Carrier et al., 1999; 2002a; 2002b). To improve cell density Radisic et al. (Radisic et al., 2003; 2008) developed a new seeding strategy within the same perfusion system as in (Carrier et al., 2002a). Using Matrigel as vehicle for cell delivery, neonatal rat CMs were seeded into collagen sponges and cultured in perfused cartridges with alternating medium flow. Initial cell densities corresponding to those normally present in the adult rat heart (about $10^8$ cells/cm$^3$) were reached, with rapid and spatially uniform cell distribution throughout the perfused constructs. The result of the direct medium perfusion (0.5 ml/min) approach was high cell viability, differentiated function of CMs and cell protection from critical hydrodynamic shear. As for the electrophysiological function, it was observed that constructs cultured in perfusion maintained constant frequency of contractions, whereas constructs cultivated in orbitally mixed dishes presented episodes resembling arrhythmia. Further studies (Dvir et al., 2007; Brown et al., 2008) demonstrated that cultivation under pulsatile interstitial fluid flow has beneficial effects on contractile properties, resulting in enhanced tissue assembly by way of mechanical conditioning and improved mass transport.

Among biomimetic strategies for *in vitro* generation of functional engineered cardiac constructs, several studies focused on the impact of electrical stimulation in enhancing functional coupling of cells and synchronously contractile tissue constructs formation. Radisic and coworkers (Radisic et al., 2004) subjected cardiac constructs cultured *in vitro* (prepared as in (Radisic et al., 2003)) to a pulsatile electrical field (rectangular, 2 ms, 5 V/cm, 1 Hz) within a glass chamber fitted with two carbon rods and

connected to a cardiac stimulator. The application of electrical stimulation induced cell alignment and coupling, and promoted the establishment of gap junctions, propagation of pacing signals and generation of action potentials that induced synchronous macroscopic contractions. Development of conductive and contractile properties of cardiac constructs was concurrent, with strong dependence on the initiation and duration of electrical stimulation (Radisic et al., 2004). Adopting a similar apparatus (Figure I.7b), further studies on electrical stimulation were performed by Tandon and coworkers (Tandon et al., 2008). Cardiac constructs were prepared as previously described (Radisic et al., 2004; 2008) and, after 3-5 days of pre-culture, electrical stimulation was performed (2 ms, 0-12.5 V/cm, 1 Hz). Contractile activity was assessed visually and ultra-structural differentiation and morphological and constitutive hallmarks of maturing CMs were observed. In more recent studies (Tandon et al., 2009a; 2011), Tandon and colleagues focused their work on the optimization of the electrical stimulation, by systematically varying stimulation parameters (electrode material, amplitude, duration, and frequency). It was found that engineered cardiac tissues stimulated with carbon electrodes (monophasic square-wave pulses, 2 ms, 3 V/cm, 3 Hz) presented the highest density, and the best-developed contractile behavior, with remarkable improvement of functional performance, cell elongation, tissue compactness and protein levels. In a further *in vitro* study, Tandon et al. (Tandon et al., 2009b) applied DC electric fields of 6 V/cm (similar to those encountered *in vivo* during development or in a post-injury phase) to human adipose and human epicardial adipose tissue-derived SCs. Upon stimulation, the following were observed: (1) cell elongation and alignment perpendicular to the applied electric field; (2) gap junctions disassembly; and (3) upregulation of the expression of genes for connexin-43, thrombomodulin, vascular endothelial growth factor, and FB growth factor.

Recently, Barash et al. (Barash et al., 2010) developed a cultivation system where perfusion and electrical stimulation were combined by inserting two carbon rod electrodes into a perfusion bioreactor. Cardiac constructs (neonatal rat cardiac cells seeded on porous alginate scaffolds) were cultured for 4 days under homogenous perfusion (25 ml/min) and continuous electrical bipolar pulse (2 ms, 5 V, 1 Hz). The combination of perfusion and electrical stimulation promoted cell elongation and striation, and enhanced expression level of connexin-43.

An approach similar to (Barash et al., 2010) was taken by Maidhof et al. (Maidhof et al., 2012), who designed a bioreactor (Figure I.7c) providing both forced perfusion and electrical stimulation to neonatal rat cardiac cells seeded on channeled PGS scaffolds. Culture medium was forced to flow downwards through the constructs placed, without the need of fixation, on a circular array of perforated holes, while electrical stimulation (monophasic square wave, 3 V/cm, 3 Hz) was obtained via two parallel carbon rod electrodes. Constructs cultured with simultaneous perfusion and electrical stimulation

exhibited substantially improved functional properties, as evidenced by a significant increase in contraction amplitude.

A growing research branch focuses on bioreactors' application for *in vitro* generation of cardiac-tissue-like 3D constructs at smaller scales. Recently, miniaturized screening platforms were developed to study the impact of physical and chemical parameters on the maturation, structure, and function of the cardiac tissue. The basic idea is to provide advanced high-throughput, low-volume *in vitro* models for drug testing and, in combination with recent induced pluripotent SC technology, disease modeling. Important requisites towards a screening platform are miniaturization, reduced manual handling, and automated readout. In 2010, Hansen et al. (Hansen et al., 2010) developed a drug screening platform based on large series of miniaturized EHTs, fabricated as strips, where the contractile activity can be automatically monitored. Neonatal rat heart cells were mixed with fibrinogen/Matrigel plus thrombin and pipetted into rectangular casting molds in which two flexible silicone posts were positioned. During cultivation, fibrin-based mini-EHTs (FBMEs) demonstrated cell spreading inside the matrix and newly formed cell-cell contacts that led to the formation of condensed FBMEs (6.3 mm length, 0.2-1.3 mm diameter) and to the imposition of direct mechanical load to cells. Elongation of cells was observed, accompanied by single cells coherent beating activity, and, after 8-10 days, FBMEs started to rhythmically deflect the posts. Analysis of a large series of FBMEs revealed high reproducibility and stability for weeks. Moreover, tests performed using drugs with known repolarization-inhibitory and cardiotoxic effects demonstrated the suitability of the FBME system as platform for *in vitro* drug screening.

Adopting the same experimental setup used for producing and monitoring FBMEs, in 2012, Schaaf et al. (Schaaf et al., 2011) generated fibrin-based human EHTs (hEHTs) from an unselected population of differentiated human ESCs containing 30-40% actinin- positive CMs. Constructs displayed a dense network of longitudinally oriented, interconnected and cross-striated CMs that allowed hEHTs to reach regular (mean 0.5 Hz) and strong (mean 0.1 mN) contractions for up to 8 weeks.

In 2011, Kensah and coworkers (Kensah et al., 2011) developed a multimodal bioreactor for mechanical stimulation of miniaturized bioartificial cardiac tissues (BCTs) and for real-time measurement of contraction forces during tissue maturation, enabling small-scale SC-based cardiac TE. Each module connected a cultivation chamber (with a glass bottom for microscopic assessment) to both a linear motor with integrated position measurement and a force sensor (measuring range of 0-1N). BCTs were prepared with neonatal rat CMs mixed with type I collagen and Matrigel, according to (Zimmermann et al., 2002a). BCTs were subjected to cyclic stretch stimulation (10%, 1 Hz) with daily real-time spontaneous active force measurement. As an end-point analysis, maximum forces were captured upon electrical stimulation of the tissues at increasing preloads (further details in Table I.2). BCTs presented spontaneous, synchronized contractions with cell orientation

along the axis of strain and a moderate increase in the systolic force (1.42 ± 0.09 mN vs. 0.96 ± 0.09 mN in controls), with a marked increase in the measured force after stimulation with noradrenalin (2.54 ± 0.11 mN). The bioreactor was designed for including additional functions such as electric pacing and culture medium perfusion. More recently, using the same bioreactor, Kensah et al. (Kensah et al., 2013) cultured highly purified murine and human pluripotent SC-derived CMs to generate functional BCTs and to investigate the role of FBs, ascorbic acid, and mechanical stimuli. For the first time, a stimulation strategy for tissue maturation was combined with a novel concept of tissue formation from non-dissociated cardiac bodies, which has led to a dramatic increase in contractile forces, comparable with native myocardium. BCTs underwent constant static stress, and an additional mechanical stretch was then applied within the bioreactor using either uniaxial cyclic stretch (10%, 1 Hz) or stepwise growing static stretch (200 µm stepwise elongation), mimicking the increasing systolic and diastolic pressure in the developing embryonic heart. Real time BCT active and passive force measurements revealed a considerably enhanced contractility of murine and human BCTs, leading to a maximum active tension of 4.4 mN/mm2 in human BCTs, only 3- to 5-fold lower than active forces reported for native human myocardium (Mulieri et al., 1992; Holubarsch et al., 1998).

In the field of scaffold design for in situ cardiac repair, the bioreactor proposed by Kensah (Kensah et al., 2011; 2013) allowed Dahlmann et al. (Dahlmann et al., 2013) to test the mechanophysical properties of a novel in situ hydrogelation system which, mimicking the native ECM, allows for the generation of contractile bioartificial cardiac tissue from CM enriched neonatal rat heart cells. The proposed in situ hydrogelation matrix is individually shapeable, exhibits adequate physical and mechanical stability, and is cytocompatible.

In 2012, Boudou et al. (Boudou et al., 2012) employed micro electro-mechanical systems (MEMS) technology to generate arrays of cardiac microtissues (CMTs) embedded within collagen/fibrin 3D micropatterned matrices. MEMS cantilevers simultaneously constrained CMT contraction and measured spontaneous contraction forces generated by the CMTs in real time. Microtissue forces were quantified by taking bright-field and fluorescence images. Electrical stimulation, obtained by placing two carbon electrodes on the sides of the samples (biphasic square pulses, 1 ms, 6 V/cm, 0.2 Hz), induced a better compaction of the matrix and a faster cell alignment, improving the cell coupling. Moreover, by forcing the CMTs to beat periodically over days, electrical stimulation increased the positive effect of the auxotonic load due to the stiff cantilevers, leading to higher cross-sectional stress. The advantage of the solution proposed by Boudou et al. (Boudou et al., 2012) is that, using a unified approach, it is possible to test the impact of mechanical preload, matrix stiffness, electrical stimulation, or soluble factors on the structural and functional properties of engineered CMTs. This could allow routine production of hundreds of functional CMTs with reproducible contractile phenotyping from readily available cardiac cells, for high-throughput, low-volume drug screening.

### *3.2 Cardiac patches*

While small infarct may be treated with cell therapy, larger areas of damaged cardiac tissue require excision and replacement with a cardiac patch. In fact, the best regeneration strategy depends on the time post-infarction; that is, new and old infarcts most likely cannot be treated using the same approach (Radisic and Sefton, 2011).

Possible approaches explored for engineering a cell-based and functional scaffolds that could be used to repair heart muscle include: (i) cell self-assembly, (ii) porous and fibrous scaffolds, (iii) cultivation of thin films (sheets) of functionally coupled cells, and (iv) cells grown on composite scaffolds (Radisic and Sefton, 2011).

In cardiac TE approaches, most studies suggest that some type of scaffold is necessary to support assembly of cardiac tissue *in vitro*. An important scaffold-free approach includes stacking of confluent monolayers of CMs (Shimizu et al., 2002). Even if cardiac patches obtained in this way generate high active force, engineering patches more than two or three layers remains a problem. Shimizu and colleagues also described the polysurgery approach. Vascularized cardiac patches can be formed by sequential layering of cell sheets in multiple surgeries (Shimizu et al., 2006). Despite this strategy demonstrates that thick tissues (~ 1 cm) can in principle be created from cell sheets, the approach will be difficult to implement in the clinical setting. Thick relevant contractile cardiac patch was obtained by combining CM cell sheets with CM-seeded small-intestinal submucosa (SIS) by Hata and colleagues. Stacked CM sheets contracted spontaneously and synchronously with seeded SIS after adherence, and a large portion of analysed constructs showed a defined contraction direction, parallel to the longitudinal axis (Hata et al., 2010). Stevens and colleagues recently managed to generate a cardiac patch based on human ESC-derived CMs by self-assembly of isolated cells in orbitally mixed dishes (Stevens et al., 2009), essentially creating cell aggregates that could be deployed as a patch.

3D cardiac tissue constructs were successfully cultivated using a variety of scaffolds amongst which collagen sponges were the most common. In the pioneering approach of Li et al., fetal rat ventricular CMs were expanded after isolation, seeded on collagen sponges, and cultivated in static dishes for up to 4 weeks (Li et al., 1999). The cells proliferated with time in culture and expressed multiple sarcomeres. Similarly, fetal cardiac cells were also cultivated on porous alginate scaffolds in static 96-well plates. After 4 days in culture the cells formed spontaneously beating aggregates in the scaffold pores (Leor et al., 2000). Cell seeding densities of the order of $10^8$ cell/$cm^3$ were achieved in the alginate scaffolds using centrifugal forces during seeding (Dar et al., 2002). Neonatal rat CMs seeded on collagen sponges formed spontaneously contracting constructs after 36h of culture (Kofidis et al., 2003) and maintained their activity for up to 12 weeks.

In a standard TE approach, fibrous PGA scaffolds were combined with neonatal rat CMs and cultivated in spinner flasks and rotating vessels (Carrier et al., 1999). The scaffold

was 97% porous and consisted of non-woven PGA fibers 14 μm in diameter. Peripheral layer of the constructs seeded with neonatal rat or embryonic chick CMs showed relatively homogeneous electrical properties and sustained macroscopically continuous impulse propagation on a centimeter-size scale (Bursac et al., 1999). Electrospun scaffolds have gained significant attention as they allow the control over structure at sub-micron levels as well as control over mechanical properties, both of which are important for cell attachment and contractile function. Zong and colleagues (Zong et al., 2005) used electrospinning to fabricate oriented biodegradable non-woven polylactic acid (PLA) scaffolds. Neonatal rat CMs cultivated on oriented PLA matrices had remarkably well-developed contractile apparatus and demonstrated electrical activity.

A significant step towards a clinically useful cardiac patch was the cultivation of ESC-derived CMs on thin polyurethane (PU) films. Cells exhibited cardiac markers (actinin) and were capable of synchronous macroscopic contractions (Alperin et al., 2005). The orientation and cell phenotype could further be improved by micro-contact printing of ECM components as demonstrated for neonatal rat CMs cultivated on thin PU and PLA films (McDevitt et al., 2002; 2003). Similarly, thin (10-15 μm diameter) several millimeter long cardiac organoids, positive for troponin I and capable to spontaneously contract, were obtained through microfluidic patterning of hyaluronic acid on glass substrates (Khademhosseini et al., 2007). In a recent study, Feinberg and colleagues seeded a layer of neonatal rat ventricular CMs on a polydimethylsiloxane (PDMS) membrane that could be detached from a thermo-sensitive layer at room temperature. Called “muscular thin films”, these cell-covered sheets could be designed to perform tasks such as gripping, and pumping by careful tailoring of the tissue architecture, this film shape, and electrical-pacing protocol (Feinberg et al., 2007). Scaffold structure can be used to effectively guide orientation of CMs and yield anisotropic structure similar to the native myocardium even in the absence of specific physical cues such as electrical or mechanical stimulation. Engelmayr et al. construct an accordion-like scaffold using laser boring of PGS layer (Engelmayr et al., 2008). The scaffolds were pre-treated with cardiac FBs followed by seeding of enriched CMs. During pre-treatment, rotating culture was used, while static culture was used upon CM seeding. Contractile cardiac grafts with mechanical properties closely resembling those of the native rat right ventricle were obtained after the culture period. In addition, the cells in the pores were aligned along the preferred direction. Bian and colleagues created a cell/fibrin hydrogel micromolding approach were PDMS molds, containing an array of elongated posts, were used to fabricate relatively large neonatal rat skeletal muscle tissue networks. As the cells compacted the hydrogel, the presence of high-aspect-ratio posts forced them to elongate and align, thus imparting a high degree of anisotropy to the cells and the tissue. This approach is currently being extended to cultivation of cardiac patches based on mouse ESC-derived progenitor cells (Bian and Bursac, 2009; Bian et al., 2009).

To combine the advantages of the presence of naturally occurring ECM (laminin) and the stability of porous scaffolds, neonatal rat CMs were inoculated into collagen sponges or synthetic PGS scaffolds using Matrigel (Radisic et al., 2006). The main advantage of a collagen sponge is that it supports cell attachment and differentiation. Nevertheless the scaffold tends to swell when placed in culture medium; making complicated the creation of a parallel channel array resembling a capillary network. For that reason a biodegradable elastomer (Wang et al., 2002) with high degree of flexibility was used. Boublik et al. have reported mechanical stimulation of hybrid cardiac grafts based on knitted hyularonic acid-based fabric and fibrin (Boublik et al., 2005). The grafts exhibited mechanical properties comparable to those of native neonatal rat hearts. In a subcutaneous rat implantation model the constructs exhibited the presence of CMs and blood vessel ingrowth after 3 weeks.

## 4. Research outline

### *4.1 Thesis objectives*

From the above discussion, it is evident that there is a clinical need to find innovative strategies in the field of cardiac TE, aiming to completely regenerate 3D damaged cardiac tissues. The objectives of this thesis, based on engineering and biological approaches in the field of cardiac TE, can be summarized as follow:

1) to develop dedicated bioreactors for cardiac TE, and in detail
   -to optimize and implement a bioreactor able to generate a biochemical and physical environment suitable for proliferation and differentiation of cells cultured on cardiac patches, by providing uniaxial cyclic stretching and electrical stimulations;
   -to design an innovative and low-cost bioreactor that, with peculiar geometric features, assures the possibility for buoyant vortices to be generate without using electromechanical rotating systems, allowing the establishment of suspension and low-shear cell culture conditions;
2) to engineer reproducible thick-relevant SM-based contractile 3D patches by using perfusion-based bioreactor culture system and type I collagen scaffold.

### *4.2 Thesis organization*

The presentation of the objectives of this thesis are organized in two parts concerning the design and development of bioreactors, and the generation of thick-relevant contractile 3D patches by using perfusion-based bioreactor culture system.

In the first part, the engineering approach, with the development of two different bioreactor prototypes, was investigated. In detail they will be illustrated: (1) a bioreactor for culturing cell-seeded cardiac patches, designed for generating a physiological biochemical and physical environment that mimics the native stimuli of the cardiac tissue, by providing uniaxial cyclic stretching and electrical stimulations; and (2) an innovative and low-cost bioreactor for providing suspension and low-shear conditions without using electromechanical rotating systems, designed for establishing a biochemical and hydrodynamic 3D culture environment suitable for maintaining specimen of different dimensions (i.e., cells, microspheres etc.) in suspension conditions.

In the second part, a biological and applicative approach was developed. In detail, the aim of this activity was to investigate the perfusion-based culture parameters suitable to engineer reproducible thick-relevant contractile 3D patches starting with mouse SMs and type I collagen scaffold.

More in detail, chapters are organized as follow.

*PART I*

*Chapter II – An electro-mechanical bioreactor providing physiological-like cardiac stimuli.*

In this chapter, the optimization and the development of an electro-mechanical bioreactor for culturing cell-seeded cardiac patches, designed for generating a physiological biochemical and physical environment that mimics the native stimuli of the cardiac tissue, by providing uniaxial cyclic stretching and electrical stimulations, was presented. The architectural design and the mechanical, electrical and control subsystems of the bioreactor were in detail presented. Finally, the results of preliminary cellular tests, demonstrating the influence of mechanical stimulation on cardiac patches, were described.

*Chapter III – An innovative suspension bioreactor.*

In this chapter, an innovative and low-cost bioreactor for providing suspension and low-shear conditions without using electromechanical rotating systems was proposed. This bioreactor is suitable for (1) testing cell/cell-microsphere constructs cytocompatibility and durability, (2) investigating the influence of suspension condition on cells cultured with or without microcarriers, and, in the late future, promoting low cost scalable cell expansion/aggregation/differentiation and long-term cell viability maintenance. The architectural design, the perfusion subsystems sizing, and computational fluid dynamic (CFD) study were in detail presented. Finally, results of preliminary cellular tests,

demonstrating the influence of suspension conditions on cardiac cells cultured on hydrogel microspheres, were described.

*PART II*

*Chapter IV – Generation of contractile 3D muscle-like tissue in a perfusion-based bioreactor culture system.*

In this chapter, the investigation of the perfusion-based culture parameters suitable to engineer reproducible thick-relevant contractile 3D patches starting with mouse SM and type I collagen scaffold was illustrated. In particular, the influence of initial cell density and of different cell culture medium composition on the ability of SMs to proliferate and differentiate into contractile units in a 3D perfusion-based bioreactor culture system were in detail described.

*Chapter V – Conclusions and future works.*

In this chapter, concluding remarks of each section were summarized with respect to the initial mission, and suggestions for future research based on the obtained results and ongoing applications were given.

## References

Akhtar S, Hughes MD, Khan A, Bibby M, Hussain M, Nawaz Q, Double J, Sayyed P. The delivery of antisense therapeutics. Advanced Drug Delivery Reviews, 2000, 44(1):3-21.

Akhyari P, Kamiya H, Haverich A, Karck M, Lichtenberg A. Myocardial tissue engineering: the extracellular matrix. European Journal of Cardiothoracic Surgery, 2008, 34(2):229–241.

Akins RE, Boyce RA, Madonna ML, Schroedl NA, Gonda SR, McLaughlin TA, Hartzell CR. Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. Tissue Engineering, 1999, 5(2):103–118.

Alperin C, Zandstra PW, Woodhouse KA. Polyurethane films seeded with embryonic stem cell-derived cardiomyocytes for use in cardiac tissue engineering applications. Biomaterials, 2005, 26(35):7377-86.

Archer R, Williams DJ. Why tissue engineering needs process engineering. Nature Biotechnology, 2005, 23(11):1353–1355.

Barash Y, Dvir T, Tandeitnik P, Ruvinov E, Guterman H, Cohen S. Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. Tissue Engineering Part C Methods, 2010, 16(6):1417–1426.

Barron V, Lyons E, Stenson-Cox C, McHugh PE, Pandit A. Bioreactors for cardiovascular cell and tissue growth: a review. Annals of Biomedical Engineering, 2003, 31(9):1017–1030.

Bian W, Liau B, Badie N, Bursac N. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. Nature Protocols, 2009, (10):1522-34.

Bian W, Bursac N. Engineered skeletal muscle tissue networks with controllable architecture. Biomaterials, 2009, 30(7):1401-12.

Bilodeau K, Mantovani D. Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review. Tissue Engineering, 2006, 12(8):2367–2383.

Birla RK, Huang YC, Dennis RG. Development of a novel bioreactor for the mechanical loading of tissue-engineered heart muscle. Tissue Engineering, 2007, 13(9):2239–2248.

Bolooki HM, Askari A. Acute Myocardial Infarction, in: Lang DM, Hobbs R, Tomecki KJ, Skugor M, Carey WD, Sekeres MA, Gordon S, Nurko S, Tavee J, Tesar GE, Dweik RA, Abelson A, Kothari S, Seballons RL (eds). Cleveland Clinic: Current Clinical Medicine. 2nd edition. Saunders. 2010. Chapter 2.

Boublik J, Park H, Radisic M, Tognana E, Chen F, Pei M, Vunjak-Novakovic G, Freed LE. Mechanical properties and remodeling of hybrid cardiac constructs made from heart cells, fibrin, and biodegradable, elastomeric knitted fabric. Tissue Engineering, 2005, 11(7-8):1122-32.

Boudou T, Legant WR, Mu A, Borochin MA, Thavandiran N, Radisic M, Zandstra PW, Epstein JA, Margulies KB, Chen CS. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Engineering Part A, 2012, 18(9–10):910–919.

Bouten CV, Dankers PY, Driessen-Mol A, Pedron S, Brizard AM, Baaijens FP. Substrates for cardiovascular tissue engineering. Advanced Drug Delivery Reviews, 2011, 63(4–5):221–241.

Brown MA, Iyer RK, Radisic M. Pulsatile perfusion bioreactor for cardiac tissue engineering. Biotechnology Progress, 2008, 24(4):907–920.

Buckberg GD. Basic science review: the helix and the heart. Journal of Thoracic and Cardiovascular Surgery, 2002, 124(5):863–883.

Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. American Journal of Physiology, 1999, 277(2 Pt 2):H433–44.

Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjak-Novakovic G. Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. Biotechnology and Bioengineering, 1999, 64(5):580–589.

Carrier RL. Cardiac Tissue Engineering: Bioreactor Cultivation Parameters. Massachusetts Institute of Technology. PhD Disseration, 2000.

Carrier RL, Rupnick M, Langer R, Schoen F, Freed LE, Vunjak-Novakovic G. Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Engineering, 2002a, 8(2):175–188.

Carrier RL, Rupnick M, Langer R, Schoen FJ, Freed LE, Vunjak-Novakovic G. Effects of oxygen on engineered cardiac muscle. Biotechnology and Bioengineering, 2002b, 78(6):617–625.

Chiu LLY, Radisic M. Cardiac tissue engineering. Current Opinion in Chemical Biology, 2013, 2(1):41-52.

Dahlmann J, Krause A, Möller L, Kensah G, Möwes M, Diekmann A, Martin U, Kirschning A, Gruh I, Dräger G. Fully defined in situ cross-linkable alginate and hyaluronic acid hydrogels for myocardial tissue engineering. Biomaterials, 2013, 34(4):940–951.

Dar A, Schachar M, Leor J, Cohen S. Cardiac tissue engineering optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. Biotechnology and Bioengineering, 2002, 80:305-312.

Devereux RB, Reichek N. Echocardiographic determination of left ventricular mass in man anatomic validation of the method. Circulation, 1977, 55(4):613–618.

Dorfman J, Duong M, Zibaitis A, Pelletier MP, Shum-Tim D, Li C, Chiu RC. Myocardial tissue engineering with autologous myoblast implantation. The Journal of Thoracic and Cardiovascular Surgery, 1998, 116(5):744-51.

Dumont K, Yperman J, Verbeken E, Segers P, Meuris B, Vandenberghe S, Flament W, Verdonk PR. Design of a new pulsatile bioreactor for tissue engineered aortic heart valve formation. Artificial Organs, 2002, 26(8):710–714.

Durand DM. Electrical stimulation of excitable tissue, in: Joseph D Bronzino (ed). The Biomedical Engineering Handbook. 2nd edition. CRC Press. 2000. Chapter 17.

Dvir T, Levy O, Shachar M, Granot Y, Cohen S. Activation of the ERK1/2 cascade via pulsatile interstitial fluid flow promotes cardiac tissue assembly. Tissue Engineering, 2007, 13(9):2185–2193.

Egli RJ, Luginbuehl R. Tissue engineering - nanomaterials in the musculoskeletal system. Swiss Medical Weekly, 2012, 142:w13647.

Elliott NT, Yuan F. A review of three-dimensional in vitro tissue models for drug discovery and transport studies. Journal of Pharmaceutical Sciences, 2011, 100(1):59-74.

Engelmayr GC Jr, Cheng M, Bettinger CJ, Borenstein JT, Langer R, Freed LE. Accordion-like honeycombs for tissue engineering of cardiac anisotropy. Nature Materials, 2008,7(12):1003-10.

Eschenhagen T, Fink C, Remmers U, Schols H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL. Three dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. Federation of American Societies for Experimental Biology Journal, 1997, 11:683–694.

Eschenhagen T, Didié M, Münzel F, Schubert P, Schneiderbanger K, Zimmermann WH. 3D engineered heart tissue for replacement therapy. Basic Research in Cardiology, 2002a, 97(Suppl 1):146–152.

Eschenhagen T, Didié M, Heubach J, Ravens U, Zimmerman WH. Cardiac tissue engineering. Transplant Immunology, 2002b, 9(2–4):315–321.

Feinberg AW, Feigel A, Shevkoplyas SS, Sheehy S, Whitesides GM, Parker KK. Muscular thin films for building actuators and powering devices. Science, 2007, 317(5843):1366-70.

Field LJ. Modulation of the cardiomyocyte cell cycle in genetically altered animals. Annals of the New York Academy of Science, 2004, 1015:160–170.

Fink C, Ergün S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. Federation of American Societies for Experimental Biology Journal, 2000, 14(5):669–679.

Fortuin NJ, Hood WP Jr, Sherman ME, Craige E. Determination of left ventricular volumes by ultrasound. Circulation, 1971, 44:575–584.

Freed LE, Guilak F, Guo XE, Gray ML, Tranquillo R, Holmes JW, Radisic M, Sefton MV, Kaplan D, Vunjak-Novakovic G. Advanced tools for tissue engineering: scaffolds, bioreactors, and signaling. Tissue Engineering, 2006, 12(12):3285–3305.

Gonen-Wadmany M, Gepstein L, Seliktar D. Controlling the cellular organization of tissue-engineered cardiac constructs. Annals of the NY Academy of Sciences, 2004, 1015:299–311.

Grayson WL, Martens TP, Eng GM, Radisic M, Vunjak-Novakovic G. Biomimetic approach to tissue engineering. Seminars in Cells and Developmental Biology, 2009, 20(6):665–673.

Habeler W, Pouillot S, Plancheron A, Pucéat M, Peschanski M, Monville C. An in vitro beating heart model for long-term assessment of experimental therapeutics. Cardiovascular Research, 2009, 81(2):253-9.

Hansen A, Eder A, Bönstrup M, Flato M, Mewe M, Schaaf S, Aksehirlioglu B, Schwörer A, Uebeler J, Eschenhagen T. Development of a Drug Screening Platform Based on Engineered Heart Tissue. Circulation Research, 2010, 107:35–44.

Hassink RJ, Dowell JD, Brutel de la Rivière A, Doevendans PA, Field LJ. Stem cell therapy for ischemic heart disease. Trends in Molecular Medicine, 2003a, 9(10):436–441.

Hassink RJ, Brutel de la Rivière A, Mummery CL, Doevendans PA. Transplantation of cells for cardiac repair. Journal of the American College of Cardiology, 2003b, 41(5):711–717.

Hassink RJ, Pasumarthi KB, Nakajima H, Rubart M, Soonpaa MH, Brutel de la Rivière A, Doevendans PA, Field LJ. Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. Cardiovascular Research, 2008, 78(1):18–25.

Hata H, Bär A, Dorfman S, Vukadinovic Z, Sawa Y, Haverich A, Hilfiker A. Engineering a novel three-dimensional contractile myocardial patch with cell sheets and decellularised matrix. European Journal Cardio-Thoracic Surgery, 2010, 38(4):450-5.

Hirt MN, Sörensen NA, Bartholdt LM, Boeddinghaus J, Schaaf S, Eder A, Vollert I, Stöhr A, Schulze T, Witten A, Stoll M, Hansen A, Eschenhagen T. Increased afterload induces pathological cardiac hypertrophy: a new in vitro model. Basic Research in Cardiology, 2012, 107(6):307.

Holubarsch C, Ludemann J, Wiessner S, Ruf T, Schulte-Baukloh H, Schmidt-Schweda S, Pieske B, Posival H, Just H. Shortening versus isometric contractions in isolated human failing and non-failing left ventricular myocardium: dependency of external work and force on muscle length, heart rate and inotropic stimulation. Cardiovascular Research, 1998, 37(1):46–57.

Jawad H, Ali NN, Lyon AR, Chen QZ, Harding SE, Boccaccini AR. Myocardial tissue engineering: a review. Journal of Tissue Engineering and Regenerative Medicine, 2007, 1(5):327–342.

Kensah G, Gruh I, Viering J, Schumann H, Dahlmann J, Meyer H, Skvorc D, Bär A, Akhyari P, Heisterkamp A, Haverich A, Martin U. A novel miniaturized multimodal bioreactor for

continuous in situ assessment of bioartificial cardiac tissue during stimulation and maturation. Tissue Engineering Part C Methods, 2011, 17(4):463–473.

Kensah G, Roa Lara A, Dahlmann J, Zweigerdt R, Schwanke K, Hegermann J, Skvorc D, Gawol A, Azizian A, Wagner S, Maier LS, Krause A, Dräger G, Ochs M, Haverich A, Gruh I, Martin U. Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. European Heart Journal, 2013, 34(15):1134-46.

Khademhosseini A, Eng G, Yeh J, Kucharczyk PA, Langer R, Vunjak-Novakovic G, Radisic M. Microfluidic patterning for fabrication of contractile cardiac organoids. Biomedical Microdevices, 2007, 9(2):149-57.

Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss D. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature, 2010, 464(7288):601–605.

Kim DH, Lipke EA, Kim P, Cheong R, Thompson S, Delannoy M, Suh KY, Tung L, Levchenko A. Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. Proceedings of the National Academy of Sciences, 2010, 107:565-570.

Klingensmith ME, Ern Chen L, Glasgow SC, Goers TA, Melby SJ. The Washington Manual of Surgery. Wolters Kluwer Health/Lippincott Williams & Wilkins. 2008.

Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. The Journal of Clinical Investigation, 1996, 98(1):216-24.

Kofidis T, Akhyari P, Wachsmann B, Mueller-Stahl K, Boublik J, Ruhparwar A, Mertsching H, Balsam L, Robbins R, Haverich A. Clinically established hemostatic scaffold (tissue fleece) as biomatrix in tissue- and organ-engineering research. Tissue Engineering, 2003, 9(3):517-23.

Korossis SA, Bolland F, Kearney JN, Fisher J, Ingham E. Bioreactors in tissue engineering, in: Nureddin Ashammakhi, Rui L Reis (eds). Topics in Tissue Engineering. Volume 2. 2005. Chapter 8.

Kuznetsova T, Herbots L, Richart T, D'hooge J, Thijs L, Fagard RH, Herregods MC, Staessen JA. Left ventricular strain and strain rate in a general population. European Heart Journal, 2008, 29(16):2014–2023.

Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium? Circulation, 2000, 102(19 Suppl 3):III56-61.

Li RK, Jia ZQ, Weisel RD, Mickle DA, Choi A, Yau TM. Survival and function of bioengineered cardiac grafts. Circulation, 1999, 100(19 Suppl):II63-9.

Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation, 1998,98(25):2800-4.

Lyons E, Pandit A. Design of bioreactors for cardiovascular applications, in: Nureddin Ashammakhi, Rui L Reis (eds). Topics in Tissue Engineering. Volume 2. 2005. Chapter 7.

Lyons F, Partap S, O'Brien FJ. Part 1: scaffolds and surfaces. Technology and Healthcare, 2008, 16(4):305–317.

Mahoney LT, Smith W, Noel MP, Florentine M, Skorton DJ, Collins SM. Measurement of right ventricular volume using cine computer tomography. Investigative Radiology, 1987, 22(6):451–455.

Maidhof R, Tandon N, Lee EJ, Luo J, Duan Y, Yeager K, Konofagou E, Vunjak-Novakovic G. Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue. Journal of Tissue Engineering and Regenerative Medicine, 2012, 6(10):e12–23.

Mann MJ, Dzau VJ. Therapeutic applications of transcription factor decoy oligonucleotides. The Journal of Clinical Investigation, 2000, 106(9): 1071–1075.

Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnology, 2004, 22(2):80–86.

Martin I, Smith T, Wendt D. Bioreactor-based roadmap for the translation of tissue engineering strategies into clinical products. Trends Biotechnology, 2009, 27(9):495–502.

Massai D, Cerino G, Gallo D, Pennella F, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and vascular disease. Journal of Healthcare Engineering, 2013, 4(3):329-70.

McDevitt TC, Angello JC, Whitney ML, Reinecke H, Hauschka SD, Murry CE, Stayton PS. In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces. Journal of Biomedical Materials Research, 2002, 60(3):472-9.

McDevitt TC, Woodhouse KA, Hauschka SD, Murry CE, Stayton PS. Spatially organized layers of cardiomyocytes on biodegradable polyurethane films for myocardial repair. Journal of Biomedical Materials Research Part A, 2003, 66(3):586-95.

Melo LG, Pachori AS, Kong D, Gnecchi M, Wang K, Pratt RE, Dzau VJ. Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium: reasons for cautious optimism. Circulation, 2004, 109(20):2386-93.

Mertsching H, Hansmann J. Bioreactor technology in cardiovascular tissue engineering. Advances in Biochemical Engineering Biotechnology, 2009, 112:29–37.

Miao W, Luo Z, Kitsis RN, Walsh K. Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. Journal of Molecular and Cellular Cardiology, 2000, 32(12):2397-402.

Mulieri LA, Hasenfuss G, Leavitt B, Allen PD, Alpert NR. Altered myocardial force-frequency relation in human heart failure. Circulation, 1992, 85:1743–1750.

Müller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, Laird PW, Kedes L. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. Journal of Molecular and Cellular Cardiology, 2002a, 34(2):107-16.

Müller-Ehmsen J, Peterson KL, Kedes L, Whittaker P, Dow JS, Long TI, Laird PW, Kloner RA. Rebuilding a damaged heart: long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. Circulation, 2002b, 105(14):1720-6.

Nag AC. Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios, 1980, 28:41–61.

Nasseri BA, Pomerantseva I, Kaazempur-Mofrad MR, Sutherland FW, Perry T, Ochoa E, Thompson CA, Mayer JE Jr, Oesterle SN, Vacanti JP. Dynamic rotational seeding and cell culture system for vascular tube formation. Tissue Engineering, 2003, 9(2):291–299.

Nuccitelli R. Endogenous ionic currents and DC electric fields in multicellular animal tissues. Bioelectromagnetics, 1992, Suppl 1:147–157.

Odedra D, Chiu L, Reis L, Rask F, Chiang K, Radisic M. Cardiac Tissue Engineering, in: Burdick JA, Mauck RL (eds). Biomaterials for Tissue Engineering Applications. A Review of the Past and Future Trends. 2011. Chapter 15.

Olmer R, Lange A, Selzer S, Kasper C, Haverich A, Martin U, Zweigerdt R. Suspension culture ofhuman pluripotent stem cells in controlled, stirred bioreactors. Tissue Engineering Part C Methods, 2012, 18(10):772–784.

Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Protocol of the National Academy of Science of the United States of America, 2001, 98(18):10344–10349.

Papadaki M, Bursac N, Langer R, Merok J, Vunjak-Novakovic G, Freed LE. Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies. American Journal of Physiology Heart and Circulatory Physiology, 2001, 280(1):168–178.

Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle regulation. Circulation Research, 2002, 90(10):1044–1054.

Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation, 1990, 81:1161–72.

Pok S, Jacot JG. Biomaterials advances in patches for congenital heart defect repair. Journal of Cardiovascular Translational Research, 2011, 4(5):646-54.

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. Science, 2011, 331(6020):1078–1080.

Pörtner R, Nagel-Heyer S, Goepfert C, Adamietz P, Meenen NM. Bioreactor design for tissue engineering. Journal for Bioscience and Bioengineering, 2005, 100(3):235–245.

Ptaszek LM, Mansour M, Ruskin JN, Chien KR. Towards regenerative therapy for cardiac disease. Lancet, 2012, 379(9819):933–942.

Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High-density seeding of myocyte cells for cardiac tissue engineering. Biotechnology and Bioengineering, 2003, 82(4):403–414.

Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. Proceedings of the National Academy of Science of the USA, 2004, 101(52):18129–18134.

Radisic M, Park H, Chen F, Salazar-Lazzaro JE, Wang Y, Dennis R, Langer R, Freed LE, Vunjak-Novakovic G. Biomimetic approach to cardiac tissue engineering: oxygen carriers and channeled scaffolds. Tissue Engineering, 2006, 12(8):2077-91.

Radisic M, Marsano A, Maidhof R, Wang Y, Vunjak-Novakovic G. Cardiac tissue engineering using perfusion bioreactor systems. Nature Protocols, 2008, 3(4):719–738.

Radisic M, Sefton MV. Cardiac Tissue, in: Atala A, Lanza R, Thomson JA, Nerem R (eds). Principles Of Regenerative Medicine Second Edition. Academic Press. 2011. Chapter 48.

Ratcliffe A, Niklason LE. Bioreactors and bioprocessing for tissue engineering. Annals of the NY Academy of Sciences, 2002, 96:210–215.

Reffelmann T, Kloner RA. Cellular cardiomyoplasty--cardiomyocytes, skeletal myoblasts, or stem cells for regenerating myocardium and treatment of heart failure? Cardiovascular Research, 2003, 58(2):358-68.

Roger VL, Go AS. Executive summary: heart disease and stroke statistics-2012 update: a report from the American Heart Association. Circulation, 2012, 125(1):188–197.

Schaaf S, Shibamiya A, Mewe M, Eder A, Stöhr A, Hirt MN, Rau T, Zimmermann WH, Conradi L, Eschenhagen T. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One, 2011, 6(10):e26397.

Schneck D. An outline of cardiovascular structure and function, in: Bernhard Palsson, Jeffrey A. Hubbell, Robert Plonsey, Joseph D. Bronzino (eds). Tissue Engineering. CRC Press. 2003. I-1–I-12.

Sen A, Kallos MS, Behie LA. New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Engineering, 2004, 10(5–6):904–913.

Severs NJ. The cardiac muscle cell. Bioessays, 2000, 22:188–99.

Shachar M, Cohen S. Cardiac tissue engineering, ex-vivo: design principles in biomaterials andbioreactors. Heart Failure Reviews, 2003, 8(3):271–276.

Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circulation Research, 2002, 90(3):e40.

Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T. Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. FASEB Journal, 2006, 20(6):708-10.

Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nature Protocols, 2009, 4(2):232-43.

Stevens KR, Pabon L, Muskheli V, Murry CE. Scaffold-free human cardiac tissue patch created from embryonic stem cells. Tissue Engineering Part A, 2009, 15(6):1211-22.

Tandon N, Marsano A, Cannizzaro C, Voldman J, Vunjak-Novakovic G. Design of electrical stimulation bioreactors for cardiac tissue engineering. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2008, 3594–3597.

Tandon N, Cannizzaro C, Chao PG, Maidhof R, Marsano A, Au HT, Radisic M, Vunjak-Novakovic G. Electrical stimulation systems for cardiac tissue engineering. Nature Protocols, 2009a, 4(2):155–173.

Tandon N, Goh B, Marsano A, Chao PH, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G. Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2009b, 6517–6521.

Tandon N, Marsano A, Maidhof R, Wan L, Park H, Vunjak-Novakovic G. Optimization of electrical stimulation parameters for cardiac tissue engineering. Journal of Tissue Engineering and Regenerative Medicine, 2011, 5(6):115–125.

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation, 2002, 105(1):93-8.

Tulloch NL, Murry CE. Trends in cardiovascular engineering: Organizing the human heart. Trends in Cardiovascular Medicine, 2013, 23(8):282-6

Venugopal JR, Prabhakaran MP, Mukherjee S, Ravichandran R, Dan K, Ramakrishna S. Biomaterial strategies for alleviation of myocardial infarction. Journal of the Royal Society Interface, 2012, 9(66):1-19.

Vunjak-Novakovic G, Tandon N, Godier A, Maidhof R, Marsano A, Martens TP, Radisic M. Challenges in cardiac tissue engineering. Tissue Engineering Part B Review, 2010, 16(2):169–187.

Wang Y, Ameer GA, Sheppard BJ, Langer R. A tough biodegradable elastomer. Nature Biotechnology, 2002, 20(6):602-6.

Weinhaus AJ, Roberts KP. Anatomy of the Human Heart, in: Iazzo PA (ed). Handbook of Cardiac Anatomy, Physiology, and Devices. 2nd Edition. 2009. Chapter 5.

Wendt D, Riboldi SA, Cioffi M, Martin I. Potential and bottlenecks of bioreactors in 3D cell culture and tissue manufacturing. Advanced Materials, 2009, 21(32–33):3352–3367.

Wikipedia. http://en.wikipedia.org/wiki/Myocardial_infarction. Accessed December, 2013.

Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. Journal of Molecular and Cellular Cardiology, 2001, 33(5):907-21.

Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnology and Bioengineering, 2000, 68(1):106–114.

Zimmermann WH, Schneiderbanger K, Schubert P, Didié M, Münzel F, Heubach JF, Kostin S, Neuhuber WL, Eschenhagen T. Tissue engineering of a differentiated cardiac muscle construct. Circulation Research, 2002a, 90(2):223–230.

Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T. Cardiac grafting of engineered heart tissue in syngenic rats. Circulation, 2002b, 106(12 Suppl 1):151–157.

Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nature Medicine, 2006, 12(4):452–458.

Zong X, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, Chu B, Entcheva E. Electrospun fine-textured scaffolds for heart tissue constructs. Biomaterials, 2005, 26(26):5330-8.

# CHAPTER I

## Introduction

## 1. Clinical motivations

Cardiac disease is the leading cause of morbidity and mortality in the Western World (Ptaszek et al., 2012; Roger and Go, 2012). The inability of fully differentiated, load-bearing cardiac tissues to *in vivo* regenerate, and the limitations of the current treatment therapies greatly motivate the urgent demand for more efficacious pharmaceuticals to treat heart disease and new methods to repair damaged cardiac tissue. Novel approaches being developed to treat heart disease include cell and gene therapy, and cardiac tissue engineering (TE) strategies (Carrier, 2000). Cell therapy, due to the inability of adult cardiomyocytes (CMs) to proliferate and regenerate injured myocardium, has emerged as an alternative treatment option. Differentiated CMs are indeed an ideal cell source for injection, since they contain a developed contractile apparatus and can integrate through gap junctions and intercalated discs with the host CMs (Radisic and Sefton, 2011). However, large numbers of clinically relevant autologous CMs are unavailable. Other alternatives for appropriate cell sources, useful for the regeneration of infarcted myocardium, have been tested in animal models by transplantation of skeletal myoblasts (SMs) (Dorfman et al., 1998), as well as CMs derived from embryonic stem cells (ESCs) (Klug et al., 1996), and bone marrow-derived mesenchymal stem cells (BM-MSCs) (Toma et al., 2002). On the other hand, gene therapy approaches based on either delivering exogenous genes capable of expressing therapeutic proteins (Losordo et al., 1998; Miao et al., 2000), or on the blockade of genes involved in the pathological process (Akhtar et al., 2000; Mann and Dzau, 2000;), are becoming alternative strategies.

However, current cell and gene therapeutic cardiac strategies suffer from limitations. Cell therapy involves the intramyocardial or coronary injection of cells suspended in an appropriate liquid (saline or culture medium). The main challenges associated with this procedure are poor survival of the injected cells (Müller-Ehmsen et al., 2002a) and washout from the injection site (Reffelmann and Kloner, 2003). According to some estimates, ~ 90% of the cells delivered through a needle leak out of the injection site (Müller-Ehmsen et al., 2002a; 2002b). In addition, a significant number of cells die within days after injection (Zhang et al., 2001; Müller-Ehmsen et al., 2002a). Thus, developing improved delivery and localization methods (e.g. hydrogels) and effective anti-death strategies could significantly improve effectiveness of cell injection procedures. Similarly, gene therapy approaches for myocardial infarction (MI), especially acute MI, are limited by the available delivery techniques. In general, in fact, the time it takes for transcription and translation is too long for a successful intervention in acute MI (Melo et al., 2004). Moreover, targeting a single gene as most commonly used in gene therapy may have conceptual limitations as well. Most pathological processes are complex and involve expression or downregulation of multiple genes. In many instances, this genetic complexity is not well understood and thus is difficult to predict a priori what the ultimate effect of

overexpressing or blockade of a single gene will be. In this respect, combination of gene and cell therapy may be a preferred approach in the treatment of heart diseases (Radisic and Sefton, 2011).

Alternatively, the innovative field of cardiac TE, aiming to generate cardiac muscle cell-constructs to be used as three-dimensional (3D) models for *in vitro* physiological and pharmacological studies and eventually for repair of damaged heart muscle *in vivo* (Carrier, 2000), could represent an effective alternative to overcome the current clinical limitations. Attempts to *in vivo* stimulate the regeneration of injured cardiac tissues were pursued on animal models by: (1) injection of differentiated cells or stem cells (SCs) in situ (Hassink et al., 2003a; 2003b); (2) mobilization of endogenous SCs with cytokines (Orlic et al, 2001); (3) activation of CM cell cycle (Pasumarthi and Field, 2002; Field, 2004) obtained, e.g., by inducing permanent coronary artery occlusion (Hassink et al., 2008) or performing apical ventricular resection (Kikuchi et al., 2010; Porrello et al., 2011); and (4) implantation of unseeded matrices. However, the application of these strategies is still limited since both providing cells with the fundamental signaling without resorting to structural supports and inducing cellular migration into unseeded implanted matrices are challenging issues (Bilodeau and Mantovani, 2006). The complexity of the scenario is augmented by the fact that mammalian hearts have a regenerative potential only for a brief period after birth that is lost during development (Porrello et al., 2011). On the contrary, *in vitro* tissue development was proven to be more effective and adaptive, with its three main components, i.e., cells, scaffolds and culture environment, that can be used individually or in combination (Lyons et al., 2008): (1) cells synthesize the new tissue; (2) scaffolds provide physical support to cells and a structural and biochemical cue tailored to promote cell adhesion, migration, proliferation and differentiation (e.g., allowing the application of physical stimuli on the engineered construct); (3) biomimetic *in vitro* culture environments, designed to replicate the *in vivo* milieu by using biologically inspired requirements, influence and drive cells to differentiate towards the desired phenotype and to express their functions, promoting extracellular matrix (ECM) formation and tissue maturation (Grayson et al., 2009; Egli and Luginbuehl, 2012).

Due to the structural and functional complexity of cardiac tissues, for the effective production of organized and functional cardiac engineered constructs *in vitro*, two fundamental requirements need to be satisfied. Firstly, cardiac TE implies the use of biomaterials that might present native-like tissue mechanical properties and/or topographical cues (Kim et al., 2010), as well as applying physiologic conditions such as perfusion (Radisic et al., 2008), electrical (Tandon et al., 2009a) and mechanical stimulation (Fink et al., 2000; Zimmermann et al., 2002a; Gonen-Wadmany et al., 2004; Birla et al., 2007) during cell culture (Chiu and Radisic, 2013, Massai et al., 2013). Consequently, a suitable dynamic environment is essential for applying these physiological conditions, and can be achieved and maintained within bioreactors, technological devices that, while monitoring

and controlling the culture environment and stimulating the construct, attempt to mimic the physiological milieu (Massai et al., 2013).

It has been demonstrated, in fact, that the possibility (1) to monitor and control the physicochemical environment, (2) to provide a wide range of physical stimuli, and, eventually, (3) to adapt culture conditions to tissue maturation through the use of bioreactor-based systems, allows to obtain engineered constructs with improved morphological and functional properties (Carrier et al., 1999; Carrier, 2000; Dumont et al., 2002; Korossis et al., 2005; Bilodeau and Mantovani, 2006). Moreover, in recent years, several studies have shown that the use of bioreactors in industrial processes for TE is sustainable both clinically and economically (Archer and Williams, 2005; Pörtner et al., 2005; Martin et al., 2009; Olmer et al., 2012), demonstrating that the use of closed, standardized, and automated systems guarantees more reproducibility and lower contamination risk than production processes carried out manually (Ratcliffe and Niklason, 2002; Shachar et al., 2003; Sen et al., 2004; Bouten et al., 2011)[1].

Moreover, cardiac TE has shown the potential to generate thick-contractile myocardium-like constructs that might be used as functional substitutes or as biological *in vitro* model systems (Bursac et al., 1999; Habeler et al, 2009; Smits et al., 2009; Hirt et al., 2012) to investigate the cardiac tissue-specific development and diseases, and to offer accurate and controlled *in vitro* tests for cell and cardiac tissue-based therapies (Tulloch and Murry, 2013), drug screening, predictive toxicology and target validation (Elliot and Yuan, 2010; Hansen et al., 2010).

Within this scenario and starting from these premises, the objectives of this work concern two fundamental aspects of the cardiac TE strategies, in particular the design and the development of dedicated bioreactors, and the generation of a thick-relevant contractile cardiac patch by using a bioreactor-based culture system.

As already discussed, the use of bioreactors allows to obtain several advantages in the attempt to mimic the physiological milieu (Bilodeau and Mantovani, 2006; Massai et al., 2013). Within this context, the developed bioreactor prototypes represent innovative support devices for cardiac TE that can be used with a dual purpose. Firstly, they can be used as model systems to investigate *in vitro* the effects of mass transport and biophysical and chemical stimuli on cardiac cell culture and cardiac tissue formation and maturation, and secondly, once optimal culture conditions have been identified, bioreactors can be used as production systems for *in vitro* generation of engineered functional cardiac tissues.

Similarly, literature data (Pok et al., 2011; Venugopal et al., 2012) report the beneficial effects of the use of patches for the injured myocardium. Within this scenario, our study provide proof-of-principle for generating a thick-relevant cardiac SM-based contractile 3D

[1] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

patch to be used as functional substitutes or as biological *in vitro* research model systems for assessing fundamental myocardial biology and physiology, and for use in pharmacological research.

The clinical motivation of this work comes from the potential clinical significance of combining these two complementary aspects of cardiac TE: in the near future, to develop innovative technological and biological tools to be used as model systems could strongly support the investigation of the still unknown mechanisms of cardiac tissue development, and in the late future, such approach could represent an effective therapeutic strategy to the reestablishment of the structure and function of injured cardiac tissues in the clinical practice, potentially contributing to the improvement of quality of life.

## 2. Anatomy, properties and pathologies of the human heart

### *2.1 The heart*

The mammalian heart is a muscular pump which serves two primary functions: (1) collect blood from the tissues of the body and pump it to the lungs; and (2) collect blood from the lungs and pump it to all other tissues in the body. The human heart lies in the protective thorax, posterior to the sternum and costal cartilages, and rests on the superior surface of the diaphragm. The heart assumes an oblique position in the thorax, with two-thirds to the left of midline. It occupies a space between the pleural cavities called the middle mediastinum, defined as the space inside of the pericardium, the covering around the heart (Figure I.1). This serous membrane has an inner and an outer layer, with a lubricating fluid in between. The fluid allows the inner visceral pericardium to "glide" against the outer parietal pericardium (Weinhaus and Roberts, 2009).

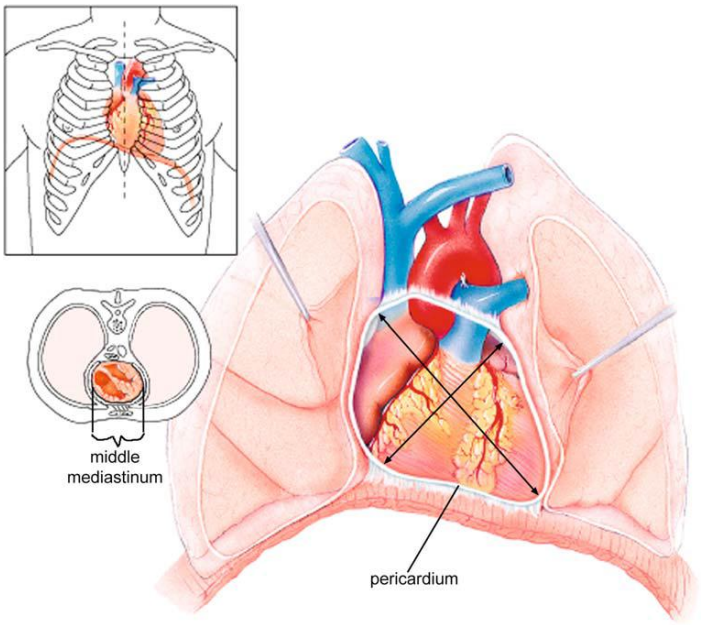


**Figure I.1.** Position of the heart in the thorax. (Weinhaus and Roberts, 2009).

The internal anatomy of the heart reveals four chambers composed of cardiac muscle or "myocardium". The two upper chambers (or atria) function mainly as collecting chambers; the two lower chambers (ventricles) are much stronger and function to pump blood. The role of the right atrium and ventricle is to collect blood from the body and pump it to the lungs. The role of the left atrium and ventricle is to collect blood from the lungs and pump it throughout the body. There is a one-way flow of blood through the heart; this flow is maintained by a set of four valves. The atrioventricular or AV valves (tricuspid and bicuspid) allow blood to flow only from atria to ventricles. The semilunar valves (pulmonary and aortic) allow blood to flow only from the ventricles out of the heart and through the great arteries (Figure I.2) (Weinhaus and Roberts, 2009).

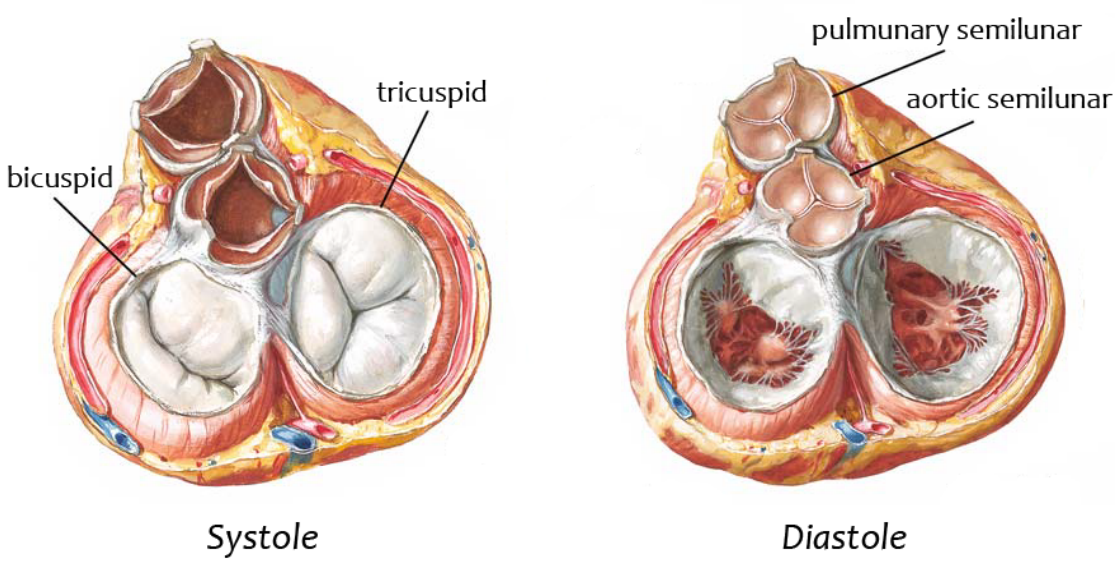


**Figure I.2.** Valves of the heart. The right AV valve is the tricuspid valve, the left is the bicuspid valve. The valve of the pulmonary trunk is the pulmonary semilunar valve, and the aortic artery has the aortic semilunar valve (Weinhaus and Roberts, 2009).

During ventricular systole, AV valves close in order to prevent the regurgitation of blood from the ventricles into the atria. During ventricular diastole, the AV valves open as the ventricles relax, and the semilunar valves close (Figure I.2). The semilunar valves prevent the backflow of blood from the great arteries into the resting ventricles.

Although the heart is filled with blood, it provides very little nourishment and oxygen to the tissues of the heart. The walls of the heart are too thick to be supplied by diffusion alone. Instead, the tissues of the heart are supplied by a separate vascular supply committed only to the heart. The arterial supply to the heart arises from the base of the aorta as the right and left coronary arteries (running in the coronary sulcus). The venous drainage is via cardiac veins that return deoxygenated blood to the right atrium. The coronary arteries arise from the ostia in the left and right sinuses of the aortic semilunar valve, course within the epicardium, and encircle the heart in the AV (coronary) and interventricular sulci (Figure I.3) (Weinhaus and Roberts, 2009).

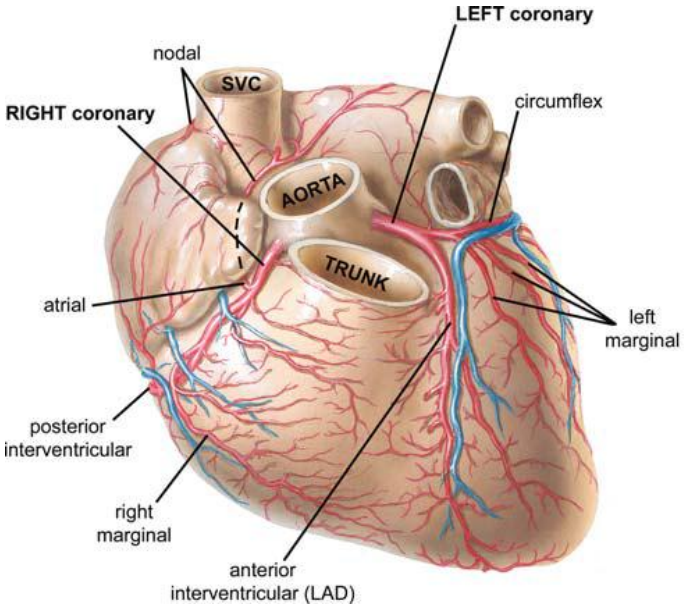


**Figure I.3.** Vascular supply to the heart. Arterial supply to the heart occurs via the right and left coronary arteries and their branches. Venous drainage occurs via cardiac veins (Weinhaus and Roberts, 2009).

A cross-section cut through the heart reveals a number of layers (Figure I.4). From superficial to deep these are: (1) the parietal pericardium with its dense fibrous layer, the fibrous pericardium; (2) the pericardial cavity (containing only serous fluid); (3) a superficial visceral pericardium or epicardium (epi = "upon" + "heart"); (4) a middle myocardium (myo="muscle"+"heart"); and (5) a deep lining called the endocardium (endo = "within"). The endocardium is the internal lining of the atrial and ventricular chambers, and is continuous with the endothelium (lining) of the incoming veins and outgoing arteries. It also covers the surfaces of the AV valves, pulmonary and aortic valves, as well as the chordae tendinae and papillary muscles. The endocardium is a sheet of epithelium called endothelium that rests on a dense connective tissue layer consisting of elastic and collagen fibers. These fibers also extend into the core of the previously mentioned valves (Weinhaus and Roberts, 2009).

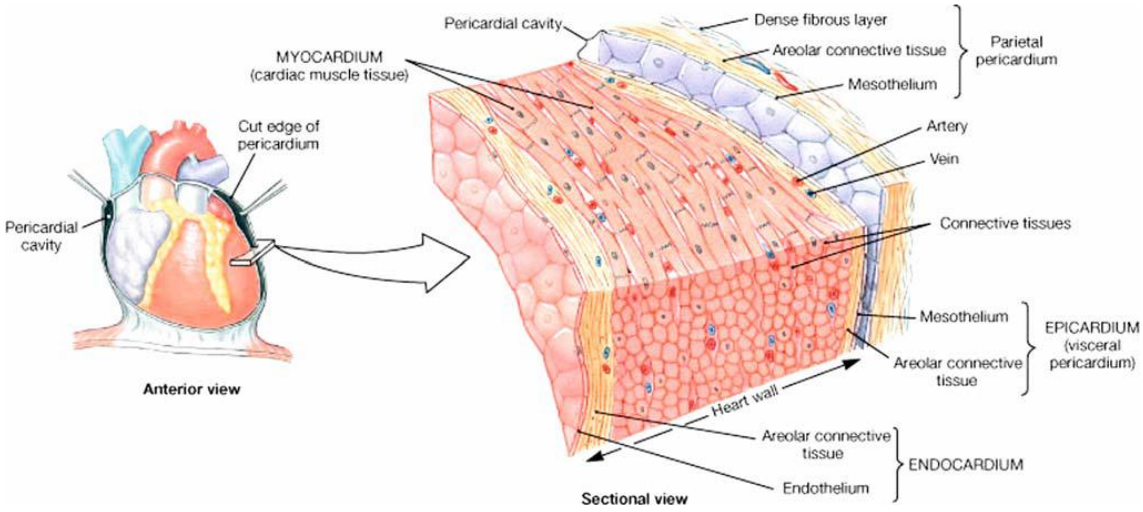


**Figure I.4.** Internal anatomy of the heart. The walls of the heart contain three layers—the superficial epicardium, the middle myocardium composed of cardiac muscle, and the inner endocardium (Weinhaus and Roberts, 2009).

The bulk of the heart tissue is the contractile myocardium, a highly differentiated tissue, ~1 cm thick in humans, with an asymmetrical and helical architecture (Buckberg, 2002; Akhyari et al., 2008), composed of tightly packed CMs (forming myofibers), fibroblasts (FBs), endothelial cells (ECs) and smooth muscle cells (SMCs). CMs are highly metabolically active; therefore oxygen and nutrients are depleted within a relatively thin layer of viable tissue (Carrier et al., 2002b). They comprise only 20–40% of the total cells in the heart but they occupy 80–90% of the heart volume (Nag, 1980). Morphologically, intact CMs have an elongated, rod shaped appearance. Contractile apparatus of CMs consists of sarcomeres arranged in parallel myofibrils (Severs, 2000). The cells are supported by a dense vasculature and an ECM that is rich in collagen and laminin. Electrical signals propagate through a 3D syncytium formed by CMs. Rapid impulse propagation is enabled by specialized junctions between cells, gap junctions, which are composed of different forms of connexin protein. The most abundant protein in ventricular CMs is connexin-43. Groups of specialized CMs (pace makers), fastest of which are located in the sinoatrial node; drive periodic contractions of the heart. Majority of the CMs in the myocardium are non-pace maker cells and they respond to the electrical stimuli generated by pace maker cells. Excitation of each CM causes an increase in the amount of cytoplasmic calcium, which triggers mechanical contraction. The result is an electrical excitation leading to a coordinated mechanical contraction to pump the blood forward (Odedra et al., 2011).

Therefore, the heart can be considered as a dynamic electromechanical system where the myocardial tissue undergoes mechanical stretch during diastole and active contraction during systole, consuming large amounts of oxygen.

The physiological stimuli that affect the entire cardiac system submit it to continuous stresses that require an enormous strength, flexibility and durability of the

structures, as well as a high degree of adaptive capacity to cope with changes due to growth, physical activity and pathological conditions (Bouten et al., 2011). In details, in the normal human heart during one cardiac cycle, the left ventricular pressure ranges between 10 and 120 mmHg, and the cavity volume varies between 40 and 130 ml (Fortuin et al., 1971; Bouten et al., 2011), respectively. The right ventricle pressure ranges between 5 to 30 mmHg (Schneck et al., 2003), and the cavity volume changes from 24 to 86.5 ml (Mahoney et al., 1987). Local mechanical loads can reach 50 kPa (Bouten et al., 2011), with 22.9% longitudinal and 59.2% radial mean strain (Kuznetsova et al., 2008). Active contraction forces of myocardial strips isolated from native human ventricles were found to range between 14.5 ± 4.4 and 22.8 ± 1.4 $mN/mm^2$ for healthy donors (Mulieri et al., 1992; Holubarsch et al., 1998). End diastolic values for the Young's modulus have been reported to range between 0.2 and 0.5 MPa (Jawad et al., 2007); however, exhaustive quantitative measures of the mechanical properties of human heart are still an open challenge.

With regard to the electrical properties, tissue in general is surrounded by extracellular fluid with relatively high electrical conductivity (3-12 mS/cm) (Durand, 2000). For vertebrates, the physiologically significant range of endogenously produced electrical field strengths is 0.1-10 V/cm (Tandon et al., 2009a). The electrical stimuli present in the heart can be classified as: (1) direct current (DC) signals, which affect and direct cell migration during the development of the cardiac primitive streak and left-right asymmetry; and (2) the pulsatile signals implicated in the development of the cardiac syncytium (Nuccitelli, 1992). In terms of frequency, the typical resting heart rate in adults is 60-100 beats per minute (bpm) that corresponds to 1-1.7 Hz (Schneck, 2003; Klingensmith et al., 2008). In terms of pulse duration, 1-2 ms is sufficiently long to excite heart tissue cells (Tandon et al., 2009a).

A summary of the heart properties and stimuli is reported in Table I.1**.**

**Table I.1.** Properties and stimuli of human heart[2].

| Property/Stimulus | Heart |
|---|---|
| **Internal diameter** | Left ventricle: 3.3-7.5 cm (Devereux and Reichek, 1977) |
| **Wall thickness** | Left ventricle: 8-15 mm (Bouten et al., 2011)<br>Right ventricle: 4-5 mm (Bouten et al., 2011) |
| **Length** | - |
| **Volume** | Left ventricle: 40-130 ml (Bouten et al., 2011; Fortuin et al., 1971)<br>Right ventricle: 24-86.5 ml (Mahoney et al., 1987) |
| **Resting rate** | 60-100 bpm (1-1.7 Hz) (Schneck, 2003; Klingensmith et al., 2008) |
| **Young's Modulus** | 0.2-0.5 MPa (end diastolic value) (Jawad et al., 2007) |
| **Pressure** | Left ventricle: 10-120 mmHg (Bouten et al., 2011)<br>Right ventricle: 5-30 mmHg (Schneck, 2003) |
| **Mechanical load** | 50 kPa (Bouten et al., 2011) |
| **Strain** | Longitudinal: 22.9% (Kuznetsova et al., 2008)<br>Radial: 59.2% (Kuznetsova et al., 2008) |
| **Electrical field** | 0.1-10 V/cm (Tandon et al., 2009a) |
| **Electrical pulse duration** | 1-2 ms (Tandon et al., 2009a) |

bpm: beats per minute

### 2.2 *The myocardial infarction*

Acute MI remains a leading cause of morbidity and mortality worldwide. MI occurs when myocardial ischemia, a diminished blood supply to the heart, exceeds a critical threshold and overwhelms myocardial cellular repair mechanisms designed to maintain normal operating function and homeostasis. Ischemia at this critical threshold level for an extended period results in irreversible myocardial cell damage or death (Bolooki and Askari, 2010).

Most MIs are caused by a disruption in the vascular endothelium associated with an unstable atherosclerotic plaque that stimulates the formation of an intracoronary thrombus, which results in coronary artery blood flow occlusion. If such an occlusion persists for more than 20 minutes, irreversible myocardial cell damage and cell death will occur (Bolooki and Askari, 2010) (Figure I.5).

[2] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

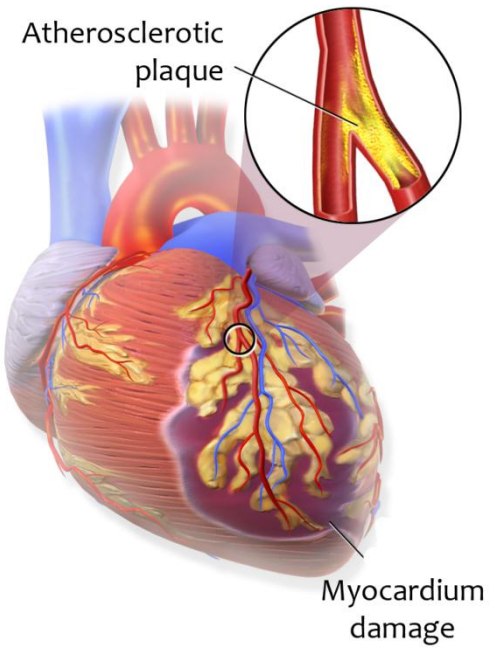


**Figure I.5.** Schematic of the MI. A MI occurs when an atherosclerotic plaque slowly builds up in the inner lining of a coronary artery and then suddenly ruptures, causing catastrophic thrombus formation, totally occluding the artery and preventing blood flow downstream
(Adapted from http://en.wikipedia.org/wiki/Myocardial_infarction).

The development of atherosclerotic plaque occurs over a period of years to decades. The two primary characteristics of the clinically symptomatic atherosclerotic plaque are a fibromuscular cap and an underlying lipid-rich core. Plaque erosion can occur because of the actions of matrix metalloproteases and the release of other collagenases and proteases in the plaque, which result in thinning of the overlying fibromuscular cap. The action of proteases, in addition to hemodynamic forces applied to the arterial segment, can lead to a disruption of the endothelium and fissuring or rupture of the fibromuscular cap. The loss of structural stability of a plaque often occurs at the juncture of the fibromuscular cap and the vessel wall, a site otherwise known as the shoulder region. Disruption of the endothelial surface can cause the formation of thrombus via platelet-mediated activation of the coagulation cascade. If a thrombus is large enough to occlude coronary blood flow, a MI can result (Bolooki and Askari, 2010).

The death of myocardial cells first occurs in the area of myocardium most distal to the arterial blood supply: the endocardium. As the duration of the occlusion increases, the area of myocardial cell death enlarges, extending from the endocardium to the myocardium and ultimately to the epicardium. The area of myocardial cell death then spreads laterally to areas of watershed or collateral perfusion. Generally, after a 6- to 8-hour period of coronary occlusion, most of the distal myocardium has died. The extent of myocardial cell death defines the magnitude of the MI. If blood flow can be restored to at-risk myocardium, more heart muscle can be saved from irreversible damage or death (Bolooki and Askari, 2010).

MI can be subcategorized on the basis of anatomic, morphologic, and diagnostic clinical information. From an anatomic or morphologic standpoint, the two types of MI are transmural and non-transmural. A transmural MI is characterized by ischemic necrosis of the full thickness of the affected muscle segment(s), extending from the endocardium through the myocardium to the epicardium. A non-transmural MI is defined as an area of ischemic necrosis that does not extend through the full thickness of myocardial wall segment(s). In a non-transmural MI, the area of ischemic necrosis is limited to the endocardium or to the endocardium and myocardium. It is the endocardial and subendocardial zones of the myocardial wall segment that are the least perfused regions of the heart and the most vulnerable to conditions of ischemia (Bolooki and Askari, 2010).

The severity of a MI depends on three factors: the level of the occlusion in the coronary artery, the length of time of the occlusion, and the presence or absence of collateral circulation. Generally, the more proximal the coronary occlusion, the more extensive the amount of myocardium that will be at risk of necrosis. The larger the MI, the greater the chance of death because of a mechanical complication or pump failure. The longer the period of vessel occlusion, the greater the chances of irreversible myocardial damage distal to the occlusion (Bolooki and Askari, 2010). As a consequence, a marked inflammatory response is elicited, developing in parallel to a dramatic decrease in nutrients and oxygen supply, and dead cells are removed by macrophages. Over the subsequent weeks to months, FBs and ECs migrate and form granulation tissue that ultimately becomes a thick and stiff collagenous scar. Scar formation reduces contractile function of the heart, heavily affects mechanical and electrical properties of native heart muscle, and leads to ventricle wall remodeling, and ultimately to heart failure (Vunjak-Novakovic et al., 2010).

Currently, there are no methods that can completely prevent these pathological events, however research is currently under way on the approaches that can stabilize the scar faster, reduce the expansion of the infarct, and decrease ventricular wall stresses that lead to further remodeling events. Treatments that limit the remodeling that occurs post infarction will improve the survival and quality of life of MI patients (Pfeffer et al., 1990).

## 3. Cardiac tissue engineering

Currently, there are a variety of methods to attenuate the effects of MI, many of which are still in the preclinical stage. Among different methods, cardiac TE is become a promising strategy that offers the possibility of creating functional tissue equivalents for scientific studies and tissue repair. The motivation for engineering cardiac muscle is two-fold: 1) a 3D culturing system may results in an easily accessible accurate *in vitro* model of natural cardiac tissue for use in physiological and pharmacological research, and 2) engineered tissue may eventually be used for the repair of damaged heart tissue. Therefore, as pointed

out in this thesis, the role of bioreactors (Chapter II and III) and scaffolds (Chapter IV), in the form of cardiac patches, is fundamental for developing cardiac TE strategies.

The following sections provide an up-to-date review of the current developed bioreactors and cardiac patches. Firstly, bioreactors for *in vitro* investigation and generation of cardiac tissues are illustrated. Afterwards, cardiac patches as biological *in vitro* model systems to investigate the cardiac tissue-specific development and diseases or as functional cardiac substitutes are described.

### *3.1 Bioreactors*[3]

Generation of tissue with complex structure and function is not feasible by culturing cells within Petri dish systems, since without the appropriate chemico-physical stimuli and 3D environment, cells cannot maintain their shapes, phenotypes, and roles, and lose the ability to proliferate and form organized tissues (Bilodeau and Mantovani, 2006). Many attempts have been made to culture cells in environments mimicking, at least partially, the *in vivo* milieu, where mechanical load, electrical stimuli, perfusion pressure, autocrine/paracrine and systemic hormonal stimulation are necessarily interwoven (Fink et al., 2000). The use of bioreactors properly designed to build up *in vitro* cell culture models allowed to study the effects of biophysical factors under closely monitored and tightly controlled culture conditions and to generate tissues *in vitro* (Martin et al., 2004; Bilodeau and Mantovani, 2006; Wendt et al., 2009). Bioreactors are closed, standardized and almost operator-independent systems assuring greater reproducibility, traceability, scalability, and lower contamination risk than traditional manual processes. Furthermore, bioreactors can be easily optimized according to present and future regulations (Ratcliffe and Niklason, 2002; Sen et al., 2004).

The use of bioreactors allows to make automated, repeatable, scalable and clinically sustainable biological processes such as: (1) cell expansion; (2) cell seeding of scaffolds; (3) cellular differentiation and tissue maturation; (4) effects of drugs on cells and engineered tissues (drug screening); and (5) *in vitro* disease model investigation. Moreover, they can be used as model systems for the investigation of cell functions and tissue development in specific environmental conditions (concentrations of oxygen, carbon dioxide, nutrients and biochemical factors; hydrodynamic conditions; physical stimuli) (Freed et al., 2006; Bouten et al., 2011). Advanced bioreactor systems should be equipped with the following (Figure I.6): (a) sensors and control systems for a real time, automatic monitoring and control of culture parameters (i.e., temperature, pH, biochemical gradients, gas concentrations, pressure, mechanical and electrical stresses, waste removal, etc.) within the culture

[3] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

chamber; (b) recirculation/perfusion systems assuring medium replacement and optimized cell and nutrient distribution within the 3D environment; and (c) physical stimulation systems for mimicking the native physiological conditions and supporting tissue maturation (Bilodeau and Mantovani, 2006; Martin et al., 2009). Therefore, for an effective *in vitro* tissue generation strategy, it is essential to define not only culture actors (cells, scaffolds, culture medium, growth factors, etc.), but also bioreactor's design criteria, which are dictated by the tissue to be produced (Barron et al., 2003; Bilodeau and Mantovani, 2006).

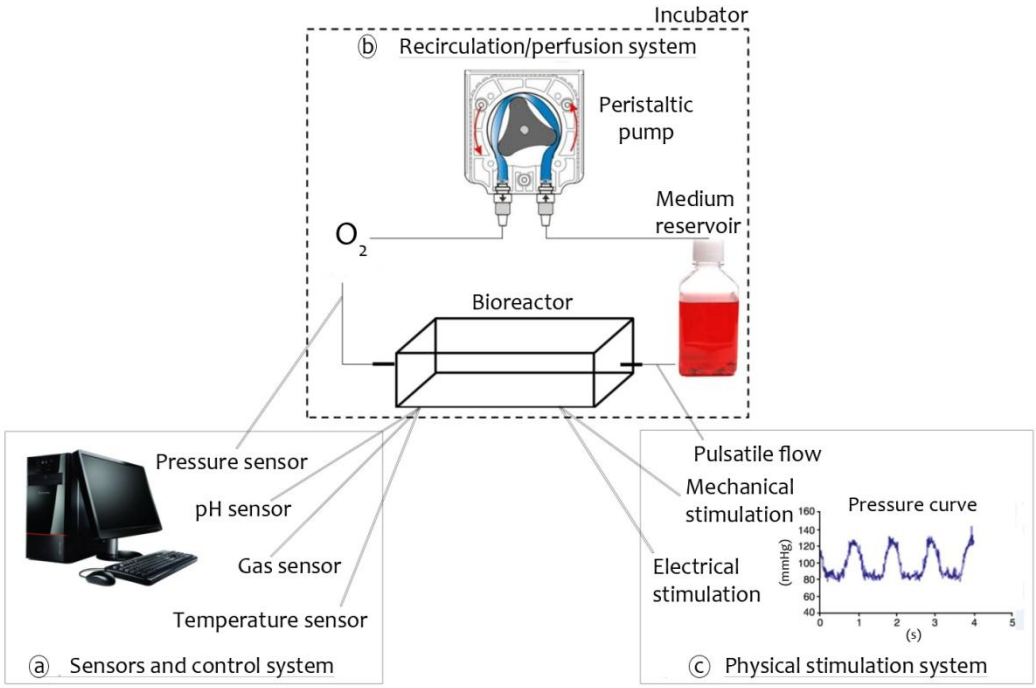


**Figure I.6.** Advanced bioreactor system equipped with (a) sensors and control systems, (b) recirculation/perfusion systems, and (c) physical stimulation systems.

Focusing on bioreactors designed for the production of cardiac constructs suitable for implantation, the presence of physical stimulation systems (i.e., mechanical stretching, electrical stimulation) is fundamental for promoting the modulation of cell behavior in terms of proliferation (Nasseri et al., 2003), differentiation (Tandon et al., 2008), protein synthesis and ECM remodeling (Mertshing and Hansmann, 2009), and for promoting structural and functional tissue maturation in terms of 3D morphology (Carrier et al., 1999), mechanical strength (Gonen-Wadmany et al., 2004) and electrical function (Tandon et al., 2009a). Hence, the complexity of the *in vivo* environment requires the fulfillment of design criteria assuring proper stimulation in terms of pulsatile forces characterized by pressures and frequency, shear stresses at physiologic frequencies (i.e., heart rate) and physiologic flow rate, stroke volume, and stroke rate values (Lyons and Pandit, 2005). An overview of the state of the art of bioreactors is provided below for the study of phenomena involved in

the mechanism of cardiac tissue formation, and for the *in vitro* generation of cardiac tissues.

### *3.1.1 Bioreactors for cardiac tissue engineering*[4]

As already discussed, the human heart has a limited capacity to regenerate itself (Papadaki et al., 2001). Therefore, the generation of 3D engineered cardiac patches to be implanted into the injured myocardium represents a challenging but effective and promising therapeutic strategy. However, cardiac TE is still in the research phase, because several problems are still to be solved to achieve full recovery of a damaged region. In detail, an engineered cardiac patch should (1) have dimensions (typically 10-50 $cm^2$ of surface area and several millimeters of thickness) and contractile features adequate to support failing hearts, thus vascularization is fundamental for its survival; (2) have a compliant response adequate to assure adaptation to systolic strength and diastolic relaxation; and (3) guarantee structural and electrical integration with the hosting myocardium (Zimmermann et al., 2006; Vunjak-Novakovic et al., 2010). The complexity of the cardiac tissue makes the fulfillment of these requirements very challenging, since adult CMs quickly dedifferentiate *in vitro* and the maintenance of their differentiation *in vitro* is still an open issue, and neonatal cells are still immature to obtain effective results from their culture. These open issues have driven the development of the biomimetic paradigm of cardiac TE, which involves the application of physiologically-relevant chemical and physical stimuli to cultured cells (Tandon et al., 2009a). Table I.2 summarizes a selection of studies, described in detail and ordered with respect to the provided physical stimuli and flow conditions, where it is demonstrated that a synergistic combination of cells, scaffolds and culture conditions within tailored bioreactors allows to obtain cardiac engineered constructs which are close to the native tissue in morphology and function, thus offering new perspectives to basic cardiac research and tissue replacement therapy.

[4] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

**Table I.2.** Devices and bioreactors for cardiac tissue engineering.

| Authors | Cells | Scaffold | Device | Physical Stimulation | Monitoring | Contraction forces |
|---|---|---|---|---|---|---|
| **Eschenhagen** *et al*., **1997** | Embryonic chick CMs | Cell/type I collagen mixture | Organ bath (static flow) | Stepwise adjusted preloads<br><br>Electrical rectangular pulse (10 ms, 20-40 V, 0.8-2 Hz) | Contractile activity | Resting tension: 1.15-2.81 mN<br><br>Twitch tension: 0.09-0.2 mN<br><br>Beating: 72.4 ± 2.6 bpm |
| **Zimmermann** *et al*., **2000** | Neonatal rat CMs | Cell/type I collagen/Matrigel mixture | Organ bath (static flow) | Stepwise adjusted preloads<br><br>Electrical rectangular pulse (5 ms, 1 Hz) | Contractile activity | Resting tension: 0.20-0.63 mN<br><br>Twitch tension: 0.21-0.46 mN<br><br>Beating: 180 bpm |
| **Fink** *et al*., **2000** | Embryonic chick CMs<br><br>Neonatal rat CMs | Cell/type I collagen/Matrigel (rat) mixture | Stretch device (static flow)<br>Organ bath (static flow) | Cyclic unidirectional stretch (1-20%, 1.5 Hz)<br><br>Stepwise adjusted preloads | Contractile activity | Force of contraction of stretched (20%) ETHs: 0.10-0.18 mN (chicken, 238% over controls) |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | Electrical rectangular pulse (10 ms, 20-40 V, 1.5 Hz (chick) or 2 Hz (rat)) | | 0.20-0.65 mN (rat, 188% over controls)<br><br>Twitch duration: decrease of 14-44% with respect to unstretched controls |
| **Zimmermann** *et al*., **2002a** | Neonatal rat CMs | Cell/type I collagen/Matrigel mixture | Stretch device (static flow) | Cyclic unidirectional stretch (10%, 2 Hz) | Contractile activity | Resting tension: 0.05-0.27 (basal condition) mN |
| | | | Organ bath (static flow) | Stepwise adjusted preloads electrical rectangular pulse (1Hz) | | Twitch tension: 0.36 (basal condition) -0.75 mN |
| **Gonen-Wadmany** *et al*., **2004** | Neonatal rat CMs and sheep aortic SMCs | Type I collagen enriched with growth factors and hormones | Mechanical stimulation bioreactor (static flow) | Cyclic strain (0-12% cyclic change in the outer diameter of each bulb, 1 Hz) | - | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| **Birla** *et al*., **2007** | Neonatal rat CMs | Fibrin gel with thrombin | Mechanical stimulation bioreactor (static flow) | Cyclic stretch (10%, 1 Hz)<br><br>Parallel electrodes (10 ms, 15 V, 1 Hz) with custom-build optical force transducer | Contractile activity | Mean specific active force: 12.1 ± 0.62 $kN/m^2$ |
| **Akins** *et al*., **1999** | Neonatal rat CMs | Fibronectin-coated polystyrene beads<br><br>Oriented collagen fibers | HARV rotating bioreactor (laminar flow)<br><br>Transparent teflon culture bags (slowly rotated) | - | Contractile frequency (real time for Teflon culture bags) | - |
| **Carrier** *et al*., **1999** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous PGA | Flsks (static and stirred turbulent flow, 50-90 rpm)<br><br>Dishes mounted on *xyz* gyrator (mixed flow, 24 rpm) | - | Culture parameters (pH, $pO_2$, $pCO_2$)<br><br>Cell damage and death (measuring medium lactate dehydrogenase | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Rotating-wall bioreactor (dynamic laminar flow, 11-12 rpm) | | Levels in supernatant) | |
| **Papadaki** *et al*., **2001** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous laminin-coated sPGA | Flasks (stirred turbulent flow, 50 rpm) dishes mounted on *xyz* gyrator (mixed flow, 25 rpm)<br><br>Rotating-wall bioreactor (dynamic laminar flow, 12 rpm) | Electrical pulses (1 ms, 0.1-5 V at a rate of 60 bpm) | Electro-physiological properties | - |
| **Carrier** *et al*., **2002a; 2002b** | Neonatal rat CMs | Porous, disk-shaped, nonwoven mesh of fibrous PGA | Flasks (stirred turbulent flow, 50 rpm)<br><br>Direct perfused cartridges (1h at 0.2 ml/min, and then 10 days at 0.6, 1 or 3 ml/min)<br><br>Electrophysiological assessment | - | Culture parameters (pH, $pO_2$, $pCO_2$) | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | apparatus | | | |
| **Radisic** *et al*., **2003** | Neonatal rat CMs (feasibility studies)<br><br>Murine C2C12 cells (detailed seeding studies) | Collagen sponges enriched with Matrigel | Orbitally mixed dishes (25 rpm)<br><br>Direct perfused cartridges (seeding: alternating flow; cultivation in a perfusion loop: unidirectional flow, 0.5 ml/min)<br><br>Electrophysiological assessment system | Electrical square pulses (2 ms, 1-5 V, 1 Hz) | Contractile activity | - |
| **Radisic** *et al*., **2004** | Neonatal rat CMs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (orbitally mixed flow, 25 rpm) | Electrical rectangular pulse (2 ms, 5 V/cm, 1 Hz) | Contractile activity (real time)<br>Electrical activity | - |
| **Tandon** *et al*., **2008; 2009a; 2011** | Neonatal rat CMs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (static flow) | Electrical square monophasic pulses (2 ms, 0-12.5 V/cm, 1 Hz) | Contractile activity (real time) | - |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| **Tandon** *et al*., **2009b** | Human adipose tissue-derived SCs<br><br>Human epicardial adipose tissue-derived SCs | Collagen sponges enriched with Matrigel | Electrical stimulation bioreactor (static flow) | Electrical DC fields (6 V/cm) | - | - |
| **Barash** *et al*., **2010** | Neonatal rat CMs | Alginate porous scaffolds | Electrical stimulation and perfusion bioreactor (25 ml/min) | Electrical bipolar pulses (2 ms, 5 V, 1 Hz) | Culture parameters (pH, $pO_2$, $pCO_2$) | - |
| **Maidhof** *et al*., **2012** | Neonatal rat cardiac cells | Channeled PGS | Perfused | Electrical square monophasic pulses (3 V/cm, 3 Hz) | Contractile activity | - |
| **Hansen** *et al*., **2010** | Neonatal rat CMs | Cell/fibrinogen/ Matrigel/ thrombin mixture | Minuaturized drug screening platform (static flow) | - | Contractile activity (real time) | Force: 0.05 to 0.4 mN (organ baths: force up to 0.9 mN, relative force up to 28.7 $mN/mm^2$) frequency: 0.3-2.7 Hz |
| **Schaaf** *et al*., **2010** | Human ESC-derived CMs | Cell/fibrinogen/ Matrigel/thrombin mixture | Miniaturized drug screening platform (static flow) | - | Contractile activity (real time) | Force: |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 0.12 mN/mm$^2$<br>Frequency:<br>0.5 Hz |
| **Kensah** *et al*., **2011** | Neonatal rat CMs | Cell/type I collagen/ Matrigel mixture | Mechanical stimulation bioreactor (static flow) | Cyclic longitudinal stretch (10%, 1 Hz) stepwise increased preloads electrical rectangular pulse (5 ms, 25 V) | Contractile activity (real time) Live cell imaging | Maximum spontaneous systolic force:<br>0.96 ± 0.09 mN (controls)<br>1.42 ± 0.09 mN (stretch alone)<br>2.54 ± 0.11 mN (noradrenalin) |
| **Kensah** *et al*., **2013** | Murine pluripotent SC-derived CMs<br><br>Human embryonic and pluripotent SC-derived CMs | Cell/Type I collagen/Matrigel mixture | Mechanical stimulation bioreactor (static flow) | Constant static stretch uniaxial cyclic Stretch (10%, 1 Hz) Growing static stretch (stepwise elongation by 200 µm) | Contractile activity (real time) Live cell imaging | Active force:<br>0.97 ± 0.07 mN (static stretch)<br>0.77 ± 0.07 mN<br><br>(cyclic stretch)<br>1.22 - 1.42 mN (ascorbic acid + growing static stretch)<br><br>Maximum specific active force: |

***(Continued)***

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | 4.4 mN/mm$^2$ (human) |
| **Boudou** *et al.*, **2012** | Neonatal rat CMs | Collagen/fibrin 3D micropatterned matrices | MEMS cantilevers | Electrical biphasic square pulses (1 ms, 6 V/cm, 0.2 Hz) | Contractile activity (real time) | - |

PGA: poly-glycolic acid; sPGA: surface-hydrolyzed PGA; PGS: poly-glycerol-sebacate; $pO_2$: partial pressure of oxygen; $pCO_2$: partial pressure of carbon dioxide; rpm: revolution per minute.

In their pioneering studies (Eschenhagen et al., 1997; Zimmermann et al., 2000; Eschenhagen et al., 2002b), Eschenhagen, Zimmermann and coworkers proposed a method for the *in vitro* production of coherently contracting 3D engineered heart tissues (EHTs) made of CMs from embryonic chicken (Eschenhagen et al., 1997) and neonatal rats (Zimmermann et al., 2000; Zimmermann et al., 2002b) mixed with collagen type I and, only when cultivating rat cells, Matrigel. Going beyond the limitations of monolayer cultures, sheet-shaped EHTs provided a simplified model suitable for the investigation of heart-like features of the constructs and for an analysis of the consequences that culture environment and genetic manipulations have on contractility. In order to measure EHT contractile forces, EHTs were then immersed in thermostated organ bathes, and subjected to stable isometric preloads and electrical pulses (more details in Table I.2). EHTs exhibited well organized myofilaments with intercellular connections, and coherent contractions after 2-3 days (Eschenhagen et al., 1997; Zimmermann et al., 2000).

The influence of chronic mechanical stretch on morphological and functional behavior of CMs was evaluated by Fink et al. (Fink et al., 2000), who subjected EHTs to phasic unidirectional stretch (1-20%, 1.5 Hz) for 6 days and then to isometric force measurement (as in (Eschenhagen et al., 1997; Zimmermann et al., 2000)). Stretched EHTs exhibited improved organization of CMs into parallel arrays of rod-shaped cells, increased cell length and width, and a marked improvement of the contractile function.

To overcome the limitation of the inhomogeneous cell distribution affecting sheetshaped EHTs, ring-shaped rat EHTs were cast (Zimmermann et al., 2002a). After 7 days of culture, ring-shaped EHTs were transferred into a modified stretch device (Figure I.7a) and submitted to unidirectional cyclic stretch (10%, 2 Hz) for 7 days; afterward, EHTs were subjected to isometric force measurements (as in (Eschenhagen et al., 1997; Zimmermann et al., 2000)). On circular EHTs, Zimmermann et al. (Zimmermann et al., 2002a) observed complexes of multicellular aggregates and longitudinally oriented cell bundles, with morphological features of adult tissue.

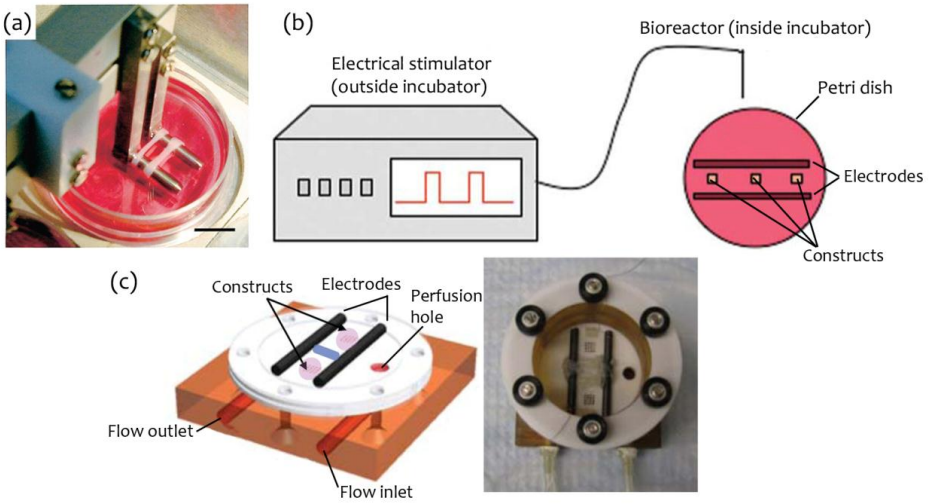


**Figure I.7.** (a) Stretch device developed by Eschenhagen (Eschemhagen et al., 2002b). (b) Bioreactor for electrical stimulation developed by Tandon (Tandon et al., 2009a). (c) Perfusion bioreactor developed by Maidhof (Maidhof et al., 2012).

Eschenhagen, Zimmermann and coworkers (Eschenhagen et al., 2002a; 2002b; Zimmermann et al., 2002b) proved the *in vivo* feasibility of the EHT implantation on rats. Implanted EHTs maintained a network of differentiated CMs and were strongly vascularized (Eschenhagen et al., 2002a; 2002b; Zimmermann et al., 2002b). Moreover, EHTs grafted on the heart of syngenic rats (Zimmermann et al., 2002b) preserved contractile function *in vivo*. More recently, Zimmermann et al. (Zimmermann et al., 2000) evaluated the performance of multiloop EHTs implanted in Wistar rat heart muscles after MI. Large force-generating EHTs were produced under elevated oxygen concentration, under auxotonic load (simultaneous changes in stress and length), and with supplementation of culture medium with insulin. To facilitate implantation, five single circular EHTs were stacked crosswise on a tailored holding device, promoting EHT fusion and allowing contractions under auxotonic load. Synchronously contracting multiloop EHTs were obtained (about 15 mm of diameter and 1–4 mm of thickness), suitable for *in vivo* engraftment. EHTs integrated well and coupled electrically to the hosting myocardium, exerting beneficial effects on systolic and diastolic left ventricular function with normalization of epicardial impulse propagation after engraftment and no evidence of arrhythmogenicity. However, proarrhythmic risks from EHT implantation are likely to be not negligible in larger, low-heart-rate, bigger species including humans.

By adopting a multi-chamber bioreactor to impart controlled simultaneous cyclic strains, Gonen-Wadmany et al. (Gonen-Wadmany et al., 2004) developed a bioartificial engineered cardiac construct (ECC) capable of synchronized multidirectional contraction. Based on previous studies (Zimmermann et al., 2000; 2002a), ECCs were prepared mixing neonatal rat CMs and sheep aortic SMCs with type I collagen gel, enriched with growth factors and hormones. ECCs, molded over silicone bulbs, were mechanically stimulated by

inflating and deflating the silicone bulbs with repetitive pneumatic pressure at 1 Hz for 6 days. Cell distribution was found to be homogeneous throughout the ECCs, and the use of SMCs resulted in a significant compaction of the collagen gels and in a cardiac substitute containing a high cell density. Moreover, the authors demonstrated that cellular and morphological reorganization is highly dependent on the amplitude of strain stimulation.

In 2007, Birla et al. (Birla et al., 2007) proposed a multi-chamber bioreactor capable of controlling tissue stimulation in order to correlate the degree of mechanical stretch to changes in the contractile performance of 3D bioengineered heart muscle (BEHM) constructs. Neonatal rat CMs were plated on fibrin-coated surfaces of cell culture plates, with the addition of thrombin, and maintained in incubator for 2 weeks. Spontaneous contractions of the primary CMs resulted in compaction of the fibrin gel promoting BEHM formation. For another 7 days, BEHMs were mechanically stimulated within the bioreactor with different time intervals per day (2, 6, and 24 hours). The active force was evaluated by stimulating the BEHMs between parallel platinum electrodes (more details in Table I.2). No apparent physical damage was found in BEHMs stretched using a stretch protocol of 10% stretch and 1 Hz, with no significant changes in the active force, specific force, pacing characteristics, or morphological features, demonstrating the structural stability of the constructs in response to applied stretch protocol.

By performing 3D suspension cultures of neonatal rat CMs on cell supports within rotating bioreactor (HARV, Synthecon), Akins et al. (Akins et al., 1999) investigated the capacity of isolated heart cells to re-establish tissue architectures *in vitro*. They observed the formation of 3D aggregates of mixed populations of ventricular cells, replicating the distribution observed *in vivo*, and presenting spontaneous and rhythmic contraction, suggesting that cardiac cells possess an innate capacity to re-establish complex 3D cardiac organization *in vitro*.

Motivated by the need to optimize seeding and perfusion of 3D scaffolds, Carrier et al. (Carrier et al., 1999) used different bioreactors (flasks, xyz gyrator, and rotating bioreactors) to investigate the *in vitro* morphogenesis of engineered cardiac muscle in a cell-polymer bioreactor model system. Porous nonwoven meshes of fibrous PGA were seeded with rat heart cells. Constructs cultivated within rotating bioreactors showed significantly improved structural and functional properties, with uniformly distributed cellularity, improved maintenance of metabolic parameters, elongated cell shape, and ultrastructural features peculiar of native cardiac tissue.

Papadaki et al. (Papadaki et al., 2001) cultivated highly concentrated neonatal rat CMs, seeded on laminin-coated PGA scaffolds, within rotating bioreactors. By using a specific apparatus (Bursac et al., 1999), they stimulated (0.1-5 V, 1 ms, pulses at a rate of 60 bpm) and recorded extracellular potentials. The engineered cardiac muscle presented a peripheral region containing CMs electrically connected through functional gap junctions. These constructs did not exhibit spontaneous beating, but responded to electrical

stimulation and showed conduction velocity of propagating electrical impulses comparable with native tissue.

However, due to the concentration gradients associated with diffusional transport of nutrients and oxygen, the engineered tissues mentioned above were limited to approximately 100-μm-thick peripheral layer around a relatively cell-free construct interior. To overcome this limitation, Carrier et al. investigated the effects of direct perfusion (Carrier et al., 2002b) and oxygen concentration (Carrier et al., 2002a) on engineered cardiac tissues obtained from fibrous PGA scaffolds seeded with neonatal rat CMs. It was found that direct medium perfusion through the constructs, located within cartridges, guarantees the following: (1) a reduction of diffusional gradients over macroscopic distances; (2) the control of local levels of pH and oxygen; (3) the spatial uniformity of cell distribution; and (4) an increase of construct thickness (Carrier et al., 2002b). It was also proven that a marked positive correlation exists between medium pO2 and the aerobicity of cell metabolism, DNA and protein content, and the expression of cardiac-specific markers (Carrier et al., 2002a). However, the system in (Carrier et al., 2002a) had two important limitations: (1) direct perfusion exposes cardiac cells to hydrodynamic shear stress values (0.2-1 dyn/cm$^2$ for perfusion rate 0.6-3 ml/min, respectively) higher than the physiological ones; (2) cell density of engineered tissue was approximately only the 20-25% of the density in native cardiac tissue (Carrier et al., 1999; 2002a; 2002b). To improve cell density Radisic et al. (Radisic et al., 2003; 2008) developed a new seeding strategy within the same perfusion system as in (Carrier et al., 2002a). Using Matrigel as vehicle for cell delivery, neonatal rat CMs were seeded into collagen sponges and cultured in perfused cartridges with alternating medium flow. Initial cell densities corresponding to those normally present in the adult rat heart (about $10^8$ cells/cm$^3$) were reached, with rapid and spatially uniform cell distribution throughout the perfused constructs. The result of the direct medium perfusion (0.5 ml/min) approach was high cell viability, differentiated function of CMs and cell protection from critical hydrodynamic shear. As for the electrophysiological function, it was observed that constructs cultured in perfusion maintained constant frequency of contractions, whereas constructs cultivated in orbitally mixed dishes presented episodes resembling arrhythmia. Further studies (Dvir et al., 2007; Brown et al., 2008) demonstrated that cultivation under pulsatile interstitial fluid flow has beneficial effects on contractile properties, resulting in enhanced tissue assembly by way of mechanical conditioning and improved mass transport.

Among biomimetic strategies for *in vitro* generation of functional engineered cardiac constructs, several studies focused on the impact of electrical stimulation in enhancing functional coupling of cells and synchronously contractile tissue constructs formation. Radisic and coworkers (Radisic et al., 2004) subjected cardiac constructs cultured *in vitro* (prepared as in (Radisic et al., 2003)) to a pulsatile electrical field (rectangular, 2 ms, 5 V/cm, 1 Hz) within a glass chamber fitted with two carbon rods and

connected to a cardiac stimulator. The application of electrical stimulation induced cell alignment and coupling, and promoted the establishment of gap junctions, propagation of pacing signals and generation of action potentials that induced synchronous macroscopic contractions. Development of conductive and contractile properties of cardiac constructs was concurrent, with strong dependence on the initiation and duration of electrical stimulation (Radisic et al., 2004). Adopting a similar apparatus (Figure I.7b), further studies on electrical stimulation were performed by Tandon and coworkers (Tandon et al., 2008). Cardiac constructs were prepared as previously described (Radisic et al., 2004; 2008) and, after 3-5 days of pre-culture, electrical stimulation was performed (2 ms, 0-12.5 V/cm, 1 Hz). Contractile activity was assessed visually and ultra-structural differentiation and morphological and constitutive hallmarks of maturing CMs were observed. In more recent studies (Tandon et al., 2009a; 2011), Tandon and colleagues focused their work on the optimization of the electrical stimulation, by systematically varying stimulation parameters (electrode material, amplitude, duration, and frequency). It was found that engineered cardiac tissues stimulated with carbon electrodes (monophasic square-wave pulses, 2 ms, 3 V/cm, 3 Hz) presented the highest density, and the best-developed contractile behavior, with remarkable improvement of functional performance, cell elongation, tissue compactness and protein levels. In a further *in vitro* study, Tandon et al. (Tandon et al., 2009b) applied DC electric fields of 6 V/cm (similar to those encountered *in vivo* during development or in a post-injury phase) to human adipose and human epicardial adipose tissue-derived SCs. Upon stimulation, the following were observed: (1) cell elongation and alignment perpendicular to the applied electric field; (2) gap junctions disassembly; and (3) upregulation of the expression of genes for connexin-43, thrombomodulin, vascular endothelial growth factor, and FB growth factor.

Recently, Barash et al. (Barash et al., 2010) developed a cultivation system where perfusion and electrical stimulation were combined by inserting two carbon rod electrodes into a perfusion bioreactor. Cardiac constructs (neonatal rat cardiac cells seeded on porous alginate scaffolds) were cultured for 4 days under homogenous perfusion (25 ml/min) and continuous electrical bipolar pulse (2 ms, 5 V, 1 Hz). The combination of perfusion and electrical stimulation promoted cell elongation and striation, and enhanced expression level of connexin-43.

An approach similar to (Barash et al., 2010) was taken by Maidhof et al. (Maidhof et al., 2012), who designed a bioreactor (Figure I.7c) providing both forced perfusion and electrical stimulation to neonatal rat cardiac cells seeded on channeled PGS scaffolds. Culture medium was forced to flow downwards through the constructs placed, without the need of fixation, on a circular array of perforated holes, while electrical stimulation (monophasic square wave, 3 V/cm, 3 Hz) was obtained via two parallel carbon rod electrodes. Constructs cultured with simultaneous perfusion and electrical stimulation

exhibited substantially improved functional properties, as evidenced by a significant increase in contraction amplitude.

A growing research branch focuses on bioreactors' application for *in vitro* generation of cardiac-tissue-like 3D constructs at smaller scales. Recently, miniaturized screening platforms were developed to study the impact of physical and chemical parameters on the maturation, structure, and function of the cardiac tissue. The basic idea is to provide advanced high-throughput, low-volume *in vitro* models for drug testing and, in combination with recent induced pluripotent SC technology, disease modeling. Important requisites towards a screening platform are miniaturization, reduced manual handling, and automated readout. In 2010, Hansen et al. (Hansen et al., 2010) developed a drug screening platform based on large series of miniaturized EHTs, fabricated as strips, where the contractile activity can be automatically monitored. Neonatal rat heart cells were mixed with fibrinogen/Matrigel plus thrombin and pipetted into rectangular casting molds in which two flexible silicone posts were positioned. During cultivation, fibrin-based mini-EHTs (FBMEs) demonstrated cell spreading inside the matrix and newly formed cell-cell contacts that led to the formation of condensed FBMEs (6.3 mm length, 0.2-1.3 mm diameter) and to the imposition of direct mechanical load to cells. Elongation of cells was observed, accompanied by single cells coherent beating activity, and, after 8-10 days, FBMEs started to rhythmically deflect the posts. Analysis of a large series of FBMEs revealed high reproducibility and stability for weeks. Moreover, tests performed using drugs with known repolarization-inhibitory and cardiotoxic effects demonstrated the suitability of the FBME system as platform for *in vitro* drug screening.

Adopting the same experimental setup used for producing and monitoring FBMEs, in 2012, Schaaf et al. (Schaaf et al., 2011) generated fibrin-based human EHTs (hEHTs) from an unselected population of differentiated human ESCs containing 30-40% actinin- positive CMs. Constructs displayed a dense network of longitudinally oriented, interconnected and cross-striated CMs that allowed hEHTs to reach regular (mean 0.5 Hz) and strong (mean 0.1 mN) contractions for up to 8 weeks.

In 2011, Kensah and coworkers (Kensah et al., 2011) developed a multimodal bioreactor for mechanical stimulation of miniaturized bioartificial cardiac tissues (BCTs) and for real-time measurement of contraction forces during tissue maturation, enabling small-scale SC-based cardiac TE. Each module connected a cultivation chamber (with a glass bottom for microscopic assessment) to both a linear motor with integrated position measurement and a force sensor (measuring range of 0-1N). BCTs were prepared with neonatal rat CMs mixed with type I collagen and Matrigel, according to (Zimmermann et al., 2002a). BCTs were subjected to cyclic stretch stimulation (10%, 1 Hz) with daily real-time spontaneous active force measurement. As an end-point analysis, maximum forces were captured upon electrical stimulation of the tissues at increasing preloads (further details in Table I.2). BCTs presented spontaneous, synchronized contractions with cell orientation

along the axis of strain and a moderate increase in the systolic force (1.42 ± 0.09 mN vs. 0.96 ± 0.09 mN in controls), with a marked increase in the measured force after stimulation with noradrenalin (2.54 ± 0.11 mN). The bioreactor was designed for including additional functions such as electric pacing and culture medium perfusion. More recently, using the same bioreactor, Kensah et al. (Kensah et al., 2013) cultured highly purified murine and human pluripotent SC-derived CMs to generate functional BCTs and to investigate the role of FBs, ascorbic acid, and mechanical stimuli. For the first time, a stimulation strategy for tissue maturation was combined with a novel concept of tissue formation from non-dissociated cardiac bodies, which has led to a dramatic increase in contractile forces, comparable with native myocardium. BCTs underwent constant static stress, and an additional mechanical stretch was then applied within the bioreactor using either uniaxial cyclic stretch (10%, 1 Hz) or stepwise growing static stretch (200 µm stepwise elongation), mimicking the increasing systolic and diastolic pressure in the developing embryonic heart. Real time BCT active and passive force measurements revealed a considerably enhanced contractility of murine and human BCTs, leading to a maximum active tension of 4.4 mN/mm2 in human BCTs, only 3- to 5-fold lower than active forces reported for native human myocardium (Mulieri et al., 1992; Holubarsch et al., 1998).

In the field of scaffold design for in situ cardiac repair, the bioreactor proposed by Kensah (Kensah et al., 2011; 2013) allowed Dahlmann et al. (Dahlmann et al., 2013) to test the mechanophysical properties of a novel in situ hydrogelation system which, mimicking the native ECM, allows for the generation of contractile bioartificial cardiac tissue from CM enriched neonatal rat heart cells. The proposed in situ hydrogelation matrix is individually shapeable, exhibits adequate physical and mechanical stability, and is cytocompatible.

In 2012, Boudou et al. (Boudou et al., 2012) employed micro electro-mechanical systems (MEMS) technology to generate arrays of cardiac microtissues (CMTs) embedded within collagen/fibrin 3D micropatterned matrices. MEMS cantilevers simultaneously constrained CMT contraction and measured spontaneous contraction forces generated by the CMTs in real time. Microtissue forces were quantified by taking bright-field and fluorescence images. Electrical stimulation, obtained by placing two carbon electrodes on the sides of the samples (biphasic square pulses, 1 ms, 6 V/cm, 0.2 Hz), induced a better compaction of the matrix and a faster cell alignment, improving the cell coupling. Moreover, by forcing the CMTs to beat periodically over days, electrical stimulation increased the positive effect of the auxotonic load due to the stiff cantilevers, leading to higher cross-sectional stress. The advantage of the solution proposed by Boudou et al. (Boudou et al., 2012) is that, using a unified approach, it is possible to test the impact of mechanical preload, matrix stiffness, electrical stimulation, or soluble factors on the structural and functional properties of engineered CMTs. This could allow routine production of hundreds of functional CMTs with reproducible contractile phenotyping from readily available cardiac cells, for high-throughput, low-volume drug screening.

### *3.2 Cardiac patches*

While small infarct may be treated with cell therapy, larger areas of damaged cardiac tissue require excision and replacement with a cardiac patch. In fact, the best regeneration strategy depends on the time post-infarction; that is, new and old infarcts most likely cannot be treated using the same approach (Radisic and Sefton, 2011).

Possible approaches explored for engineering a cell-based and functional scaffolds that could be used to repair heart muscle include: (i) cell self-assembly, (ii) porous and fibrous scaffolds, (iii) cultivation of thin films (sheets) of functionally coupled cells, and (iv) cells grown on composite scaffolds (Radisic and Sefton, 2011).

In cardiac TE approaches, most studies suggest that some type of scaffold is necessary to support assembly of cardiac tissue *in vitro*. An important scaffold-free approach includes stacking of confluent monolayers of CMs (Shimizu et al., 2002). Even if cardiac patches obtained in this way generate high active force, engineering patches more than two or three layers remains a problem. Shimizu and colleagues also described the polysurgery approach. Vascularized cardiac patches can be formed by sequential layering of cell sheets in multiple surgeries (Shimizu et al., 2006). Despite this strategy demonstrates that thick tissues (~ 1 cm) can in principle be created from cell sheets, the approach will be difficult to implement in the clinical setting. Thick relevant contractile cardiac patch was obtained by combining CM cell sheets with CM-seeded small-intestinal submucosa (SIS) by Hata and colleagues. Stacked CM sheets contracted spontaneously and synchronously with seeded SIS after adherence, and a large portion of analysed constructs showed a defined contraction direction, parallel to the longitudinal axis (Hata et al., 2010). Stevens and colleagues recently managed to generate a cardiac patch based on human ESC-derived CMs by self-assembly of isolated cells in orbitally mixed dishes (Stevens et al., 2009), essentially creating cell aggregates that could be deployed as a patch.

3D cardiac tissue constructs were successfully cultivated using a variety of scaffolds amongst which collagen sponges were the most common. In the pioneering approach of Li et al., fetal rat ventricular CMs were expanded after isolation, seeded on collagen sponges, and cultivated in static dishes for up to 4 weeks (Li et al., 1999). The cells proliferated with time in culture and expressed multiple sarcomeres. Similarly, fetal cardiac cells were also cultivated on porous alginate scaffolds in static 96-well plates. After 4 days in culture the cells formed spontaneously beating aggregates in the scaffold pores (Leor et al., 2000). Cell seeding densities of the order of $10^8$ cell/$cm^3$ were achieved in the alginate scaffolds using centrifugal forces during seeding (Dar et al., 2002). Neonatal rat CMs seeded on collagen sponges formed spontaneously contracting constructs after 36h of culture (Kofidis et al., 2003) and maintained their activity for up to 12 weeks.

In a standard TE approach, fibrous PGA scaffolds were combined with neonatal rat CMs and cultivated in spinner flasks and rotating vessels (Carrier et al., 1999). The scaffold

was 97% porous and consisted of non-woven PGA fibers 14 μm in diameter. Peripheral layer of the constructs seeded with neonatal rat or embryonic chick CMs showed relatively homogeneous electrical properties and sustained macroscopically continuous impulse propagation on a centimeter-size scale (Bursac et al., 1999). Electrospun scaffolds have gained significant attention as they allow the control over structure at sub-micron levels as well as control over mechanical properties, both of which are important for cell attachment and contractile function. Zong and colleagues (Zong et al., 2005) used electrospinning to fabricate oriented biodegradable non-woven polylactic acid (PLA) scaffolds. Neonatal rat CMs cultivated on oriented PLA matrices had remarkably well-developed contractile apparatus and demonstrated electrical activity.

A significant step towards a clinically useful cardiac patch was the cultivation of ESC-derived CMs on thin polyurethane (PU) films. Cells exhibited cardiac markers (actinin) and were capable of synchronous macroscopic contractions (Alperin et al., 2005). The orientation and cell phenotype could further be improved by micro-contact printing of ECM components as demonstrated for neonatal rat CMs cultivated on thin PU and PLA films (McDevitt et al., 2002; 2003). Similarly, thin (10-15 μm diameter) several millimeter long cardiac organoids, positive for troponin I and capable to spontaneously contract, were obtained through microfluidic patterning of hyaluronic acid on glass substrates (Khademhosseini et al., 2007). In a recent study, Feinberg and colleagues seeded a layer of neonatal rat ventricular CMs on a polydimethylsiloxane (PDMS) membrane that could be detached from a thermo-sensitive layer at room temperature. Called “muscular thin films”, these cell-covered sheets could be designed to perform tasks such as gripping, and pumping by careful tailoring of the tissue architecture, this film shape, and electrical-pacing protocol (Feinberg et al., 2007). Scaffold structure can be used to effectively guide orientation of CMs and yield anisotropic structure similar to the native myocardium even in the absence of specific physical cues such as electrical or mechanical stimulation. Engelmayr et al. construct an accordion-like scaffold using laser boring of PGS layer (Engelmayr et al., 2008). The scaffolds were pre-treated with cardiac FBs followed by seeding of enriched CMs. During pre-treatment, rotating culture was used, while static culture was used upon CM seeding. Contractile cardiac grafts with mechanical properties closely resembling those of the native rat right ventricle were obtained after the culture period. In addition, the cells in the pores were aligned along the preferred direction. Bian and colleagues created a cell/fibrin hydrogel micromolding approach were PDMS molds, containing an array of elongated posts, were used to fabricate relatively large neonatal rat skeletal muscle tissue networks. As the cells compacted the hydrogel, the presence of high-aspect-ratio posts forced them to elongate and align, thus imparting a high degree of anisotropy to the cells and the tissue. This approach is currently being extended to cultivation of cardiac patches based on mouse ESC-derived progenitor cells (Bian and Bursac, 2009; Bian et al., 2009).

To combine the advantages of the presence of naturally occurring ECM (laminin) and the stability of porous scaffolds, neonatal rat CMs were inoculated into collagen sponges or synthetic PGS scaffolds using Matrigel (Radisic et al., 2006). The main advantage of a collagen sponge is that it supports cell attachment and differentiation. Nevertheless the scaffold tends to swell when placed in culture medium; making complicated the creation of a parallel channel array resembling a capillary network. For that reason a biodegradable elastomer (Wang et al., 2002) with high degree of flexibility was used. Boublik et al. have reported mechanical stimulation of hybrid cardiac grafts based on knitted hyularonic acid-based fabric and fibrin (Boublik et al., 2005). The grafts exhibited mechanical properties comparable to those of native neonatal rat hearts. In a subcutaneous rat implantation model the constructs exhibited the presence of CMs and blood vessel ingrowth after 3 weeks.

## 4. Research outline

### *4.1 Thesis objectives*

From the above discussion, it is evident that there is a clinical need to find innovative strategies in the field of cardiac TE, aiming to completely regenerate 3D damaged cardiac tissues. The objectives of this thesis, based on engineering and biological approaches in the field of cardiac TE, can be summarized as follow:

1) to develop dedicated bioreactors for cardiac TE, and in detail
   -to optimize and implement a bioreactor able to generate a biochemical and physical environment suitable for proliferation and differentiation of cells cultured on cardiac patches, by providing uniaxial cyclic stretching and electrical stimulations;
   -to design an innovative and low-cost bioreactor that, with peculiar geometric features, assures the possibility for buoyant vortices to be generate without using electromechanical rotating systems, allowing the establishment of suspension and low-shear cell culture conditions;
2) to engineer reproducible thick-relevant SM-based contractile 3D patches by using perfusion-based bioreactor culture system and type I collagen scaffold.

### *4.2 Thesis organization*

The presentation of the objectives of this thesis are organized in two parts concerning the design and development of bioreactors, and the generation of thick-relevant contractile 3D patches by using perfusion-based bioreactor culture system.

In the first part, the engineering approach, with the development of two different bioreactor prototypes, was investigated. In detail they will be illustrated: (1) a bioreactor for culturing cell-seeded cardiac patches, designed for generating a physiological biochemical and physical environment that mimics the native stimuli of the cardiac tissue, by providing uniaxial cyclic stretching and electrical stimulations; and (2) an innovative and low-cost bioreactor for providing suspension and low-shear conditions without using electromechanical rotating systems, designed for establishing a biochemical and hydrodynamic 3D culture environment suitable for maintaining specimen of different dimensions (i.e., cells, microspheres etc.) in suspension conditions.

In the second part, a biological and applicative approach was developed. In detail, the aim of this activity was to investigate the perfusion-based culture parameters suitable to engineer reproducible thick-relevant contractile 3D patches starting with mouse SMs and type I collagen scaffold.

More in detail, chapters are organized as follow.

*PART I*

*Chapter II – An electro-mechanical bioreactor providing physiological-like cardiac stimuli.*

In this chapter, the optimization and the development of an electro-mechanical bioreactor for culturing cell-seeded cardiac patches, designed for generating a physiological biochemical and physical environment that mimics the native stimuli of the cardiac tissue, by providing uniaxial cyclic stretching and electrical stimulations, was presented. The architectural design and the mechanical, electrical and control subsystems of the bioreactor were in detail presented. Finally, the results of preliminary cellular tests, demonstrating the influence of mechanical stimulation on cardiac patches, were described.

*Chapter III – An innovative suspension bioreactor.*

In this chapter, an innovative and low-cost bioreactor for providing suspension and low-shear conditions without using electromechanical rotating systems was proposed. This bioreactor is suitable for (1) testing cell/cell-microsphere constructs cytocompatibility and durability, (2) investigating the influence of suspension condition on cells cultured with or without microcarriers, and, in the late future, promoting low cost scalable cell expansion/aggregation/differentiation and long-term cell viability maintenance. The architectural design, the perfusion subsystems sizing, and computational fluid dynamic (CFD) study were in detail presented. Finally, results of preliminary cellular tests,

demonstrating the influence of suspension conditions on cardiac cells cultured on hydrogel microspheres, were described.

*PART II*

*Chapter IV – Generation of contractile 3D muscle-like tissue in a perfusion-based bioreactor culture system.*

In this chapter, the investigation of the perfusion-based culture parameters suitable to engineer reproducible thick-relevant contractile 3D patches starting with mouse SM and type I collagen scaffold was illustrated. In particular, the influence of initial cell density and of different cell culture medium composition on the ability of SMs to proliferate and differentiate into contractile units in a 3D perfusion-based bioreactor culture system were in detail described.

*Chapter V – Conclusions and future works.*

In this chapter, concluding remarks of each section were summarized with respect to the initial mission, and suggestions for future research based on the obtained results and ongoing applications were given.

## References

Akhtar S, Hughes MD, Khan A, Bibby M, Hussain M, Nawaz Q, Double J, Sayyed P. The delivery of antisense therapeutics. Advanced Drug Delivery Reviews, 2000, 44(1):3-21.

Akhyari P, Kamiya H, Haverich A, Karck M, Lichtenberg A. Myocardial tissue engineering: the extracellular matrix. European Journal of Cardiothoracic Surgery, 2008, 34(2):229–241.

Akins RE, Boyce RA, Madonna ML, Schroedl NA, Gonda SR, McLaughlin TA, Hartzell CR. Cardiac organogenesis in vitro: reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. Tissue Engineering, 1999, 5(2):103–118.

Alperin C, Zandstra PW, Woodhouse KA. Polyurethane films seeded with embryonic stem cell-derived cardiomyocytes for use in cardiac tissue engineering applications. Biomaterials, 2005, 26(35):7377-86.

Archer R, Williams DJ. Why tissue engineering needs process engineering. Nature Biotechnology, 2005, 23(11):1353–1355.

Barash Y, Dvir T, Tandeitnik P, Ruvinov E, Guterman H, Cohen S. Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. Tissue Engineering Part C Methods, 2010, 16(6):1417–1426.

Barron V, Lyons E, Stenson-Cox C, McHugh PE, Pandit A. Bioreactors for cardiovascular cell and tissue growth: a review. Annals of Biomedical Engineering, 2003, 31(9):1017–1030.

Bian W, Liau B, Badie N, Bursac N. Mesoscopic hydrogel molding to control the 3D geometry of bioartificial muscle tissues. Nature Protocols, 2009, (10):1522-34.

Bian W, Bursac N. Engineered skeletal muscle tissue networks with controllable architecture. Biomaterials, 2009, 30(7):1401-12.

Bilodeau K, Mantovani D. Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review. Tissue Engineering, 2006, 12(8):2367–2383.

Birla RK, Huang YC, Dennis RG. Development of a novel bioreactor for the mechanical loading of tissue-engineered heart muscle. Tissue Engineering, 2007, 13(9):2239–2248.

Bolooki HM, Askari A. Acute Myocardial Infarction, in: Lang DM, Hobbs R, Tomecki KJ, Skugor M, Carey WD, Sekeres MA, Gordon S, Nurko S, Tavee J, Tesar GE, Dweik RA, Abelson A, Kothari S, Seballons RL (eds). Cleveland Clinic: Current Clinical Medicine. 2nd edition. Saunders. 2010. Chapter 2.

Boublik J, Park H, Radisic M, Tognana E, Chen F, Pei M, Vunjak-Novakovic G, Freed LE. Mechanical properties and remodeling of hybrid cardiac constructs made from heart cells, fibrin, and biodegradable, elastomeric knitted fabric. Tissue Engineering, 2005, 11(7-8):1122-32.

Boudou T, Legant WR, Mu A, Borochin MA, Thavandiran N, Radisic M, Zandstra PW, Epstein JA, Margulies KB, Chen CS. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Engineering Part A, 2012, 18(9–10):910–919.

Bouten CV, Dankers PY, Driessen-Mol A, Pedron S, Brizard AM, Baaijens FP. Substrates for cardiovascular tissue engineering. Advanced Drug Delivery Reviews, 2011, 63(4–5):221–241.

Brown MA, Iyer RK, Radisic M. Pulsatile perfusion bioreactor for cardiac tissue engineering. Biotechnology Progress, 2008, 24(4):907–920.

Buckberg GD. Basic science review: the helix and the heart. Journal of Thoracic and Cardiovascular Surgery, 2002, 124(5):863–883.

Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. American Journal of Physiology, 1999, 277(2 Pt 2):H433–44.

Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE, Vunjak-Novakovic G. Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization. Biotechnology and Bioengineering, 1999, 64(5):580–589.

Carrier RL. Cardiac Tissue Engineering: Bioreactor Cultivation Parameters. Massachusetts Institute of Technology. PhD Disseration, 2000.

Carrier RL, Rupnick M, Langer R, Schoen F, Freed LE, Vunjak-Novakovic G. Perfusion improves tissue architecture of engineered cardiac muscle. Tissue Engineering, 2002a, 8(2):175–188.

Carrier RL, Rupnick M, Langer R, Schoen FJ, Freed LE, Vunjak-Novakovic G. Effects of oxygen on engineered cardiac muscle. Biotechnology and Bioengineering, 2002b, 78(6):617–625.

Chiu LLY, Radisic M. Cardiac tissue engineering. Current Opinion in Chemical Biology, 2013, 2(1):41-52.

Dahlmann J, Krause A, Möller L, Kensah G, Möwes M, Diekmann A, Martin U, Kirschning A, Gruh I, Dräger G. Fully defined in situ cross-linkable alginate and hyaluronic acid hydrogels for myocardial tissue engineering. Biomaterials, 2013, 34(4):940–951.

Dar A, Schachar M, Leor J, Cohen S. Cardiac tissue engineering optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. Biotechnology and Bioengineering, 2002, 80:305-312.

Devereux RB, Reichek N. Echocardiographic determination of left ventricular mass in man anatomic validation of the method. Circulation, 1977, 55(4):613–618.

Dorfman J, Duong M, Zibaitis A, Pelletier MP, Shum-Tim D, Li C, Chiu RC. Myocardial tissue engineering with autologous myoblast implantation. The Journal of Thoracic and Cardiovascular Surgery, 1998, 116(5):744-51.

Dumont K, Yperman J, Verbeken E, Segers P, Meuris B, Vandenberghe S, Flament W, Verdonk PR. Design of a new pulsatile bioreactor for tissue engineered aortic heart valve formation. Artificial Organs, 2002, 26(8):710–714.

Durand DM. Electrical stimulation of excitable tissue, in: Joseph D Bronzino (ed). The Biomedical Engineering Handbook. 2nd edition. CRC Press. 2000. Chapter 17.

Dvir T, Levy O, Shachar M, Granot Y, Cohen S. Activation of the ERK1/2 cascade via pulsatile interstitial fluid flow promotes cardiac tissue assembly. Tissue Engineering, 2007, 13(9):2185–2193.

Egli RJ, Luginbuehl R. Tissue engineering - nanomaterials in the musculoskeletal system. Swiss Medical Weekly, 2012, 142:w13647.

Elliott NT, Yuan F. A review of three-dimensional in vitro tissue models for drug discovery and transport studies. Journal of Pharmaceutical Sciences, 2011, 100(1):59-74.

Engelmayr GC Jr, Cheng M, Bettinger CJ, Borenstein JT, Langer R, Freed LE. Accordion-like honeycombs for tissue engineering of cardiac anisotropy. Nature Materials, 2008,7(12):1003-10.

Eschenhagen T, Fink C, Remmers U, Schols H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL. Three dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. Federation of American Societies for Experimental Biology Journal, 1997, 11:683–694.

Eschenhagen T, Didié M, Münzel F, Schubert P, Schneiderbanger K, Zimmermann WH. 3D engineered heart tissue for replacement therapy. Basic Research in Cardiology, 2002a, 97(Suppl 1):146–152.

Eschenhagen T, Didié M, Heubach J, Ravens U, Zimmerman WH. Cardiac tissue engineering. Transplant Immunology, 2002b, 9(2–4):315–321.

Feinberg AW, Feigel A, Shevkoplyas SS, Sheehy S, Whitesides GM, Parker KK. Muscular thin films for building actuators and powering devices. Science, 2007, 317(5843):1366-70.

Field LJ. Modulation of the cardiomyocyte cell cycle in genetically altered animals. Annals of the New York Academy of Science, 2004, 1015:160–170.

Fink C, Ergün S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. Federation of American Societies for Experimental Biology Journal, 2000, 14(5):669–679.

Fortuin NJ, Hood WP Jr, Sherman ME, Craige E. Determination of left ventricular volumes by ultrasound. Circulation, 1971, 44:575–584.

Freed LE, Guilak F, Guo XE, Gray ML, Tranquillo R, Holmes JW, Radisic M, Sefton MV, Kaplan D, Vunjak-Novakovic G. Advanced tools for tissue engineering: scaffolds, bioreactors, and signaling. Tissue Engineering, 2006, 12(12):3285–3305.

Gonen-Wadmany M, Gepstein L, Seliktar D. Controlling the cellular organization of tissue-engineered cardiac constructs. Annals of the NY Academy of Sciences, 2004, 1015:299–311.

Grayson WL, Martens TP, Eng GM, Radisic M, Vunjak-Novakovic G. Biomimetic approach to tissue engineering. Seminars in Cells and Developmental Biology, 2009, 20(6):665–673.

Habeler W, Pouillot S, Plancheron A, Pucéat M, Peschanski M, Monville C. An in vitro beating heart model for long-term assessment of experimental therapeutics. Cardiovascular Research, 2009, 81(2):253-9.

Hansen A, Eder A, Bönstrup M, Flato M, Mewe M, Schaaf S, Aksehirlioglu B, Schwörer A, Uebeler J, Eschenhagen T. Development of a Drug Screening Platform Based on Engineered Heart Tissue. Circulation Research, 2010, 107:35–44.

Hassink RJ, Dowell JD, Brutel de la Rivière A, Doevendans PA, Field LJ. Stem cell therapy for ischemic heart disease. Trends in Molecular Medicine, 2003a, 9(10):436–441.

Hassink RJ, Brutel de la Rivière A, Mummery CL, Doevendans PA. Transplantation of cells for cardiac repair. Journal of the American College of Cardiology, 2003b, 41(5):711–717.

Hassink RJ, Pasumarthi KB, Nakajima H, Rubart M, Soonpaa MH, Brutel de la Rivière A, Doevendans PA, Field LJ. Cardiomyocyte cell cycle activation improves cardiac function after myocardial infarction. Cardiovascular Research, 2008, 78(1):18–25.

Hata H, Bär A, Dorfman S, Vukadinovic Z, Sawa Y, Haverich A, Hilfiker A. Engineering a novel three-dimensional contractile myocardial patch with cell sheets and decellularised matrix. European Journal Cardio-Thoracic Surgery, 2010, 38(4):450-5.

Hirt MN, Sörensen NA, Bartholdt LM, Boeddinghaus J, Schaaf S, Eder A, Vollert I, Stöhr A, Schulze T, Witten A, Stoll M, Hansen A, Eschenhagen T. Increased afterload induces pathological cardiac hypertrophy: a new in vitro model. Basic Research in Cardiology, 2012, 107(6):307.

Holubarsch C, Ludemann J, Wiessner S, Ruf T, Schulte-Baukloh H, Schmidt-Schweda S, Pieske B, Posival H, Just H. Shortening versus isometric contractions in isolated human failing and non-failing left ventricular myocardium: dependency of external work and force on muscle length, heart rate and inotropic stimulation. Cardiovascular Research, 1998, 37(1):46–57.

Jawad H, Ali NN, Lyon AR, Chen QZ, Harding SE, Boccaccini AR. Myocardial tissue engineering: a review. Journal of Tissue Engineering and Regenerative Medicine, 2007, 1(5):327–342.

Kensah G, Gruh I, Viering J, Schumann H, Dahlmann J, Meyer H, Skvorc D, Bär A, Akhyari P, Heisterkamp A, Haverich A, Martin U. A novel miniaturized multimodal bioreactor for

continuous in situ assessment of bioartificial cardiac tissue during stimulation and maturation. Tissue Engineering Part C Methods, 2011, 17(4):463–473.

Kensah G, Roa Lara A, Dahlmann J, Zweigerdt R, Schwanke K, Hegermann J, Skvorc D, Gawol A, Azizian A, Wagner S, Maier LS, Krause A, Dräger G, Ochs M, Haverich A, Gruh I, Martin U. Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. European Heart Journal, 2013, 34(15):1134-46.

Khademhosseini A, Eng G, Yeh J, Kucharczyk PA, Langer R, Vunjak-Novakovic G, Radisic M. Microfluidic patterning for fabrication of contractile cardiac organoids. Biomedical Microdevices, 2007, 9(2):149-57.

Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY, Poss D. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature, 2010, 464(7288):601–605.

Kim DH, Lipke EA, Kim P, Cheong R, Thompson S, Delannoy M, Suh KY, Tung L, Levchenko A. Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. Proceedings of the National Academy of Sciences, 2010, 107:565-570.

Klingensmith ME, Ern Chen L, Glasgow SC, Goers TA, Melby SJ. The Washington Manual of Surgery. Wolters Kluwer Health/Lippincott Williams & Wilkins. 2008.

Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. The Journal of Clinical Investigation, 1996, 98(1):216-24.

Kofidis T, Akhyari P, Wachsmann B, Mueller-Stahl K, Boublik J, Ruhparwar A, Mertsching H, Balsam L, Robbins R, Haverich A. Clinically established hemostatic scaffold (tissue fleece) as biomatrix in tissue- and organ-engineering research. Tissue Engineering, 2003, 9(3):517-23.

Korossis SA, Bolland F, Kearney JN, Fisher J, Ingham E. Bioreactors in tissue engineering, in: Nureddin Ashammakhi, Rui L Reis (eds). Topics in Tissue Engineering. Volume 2. 2005. Chapter 8.

Kuznetsova T, Herbots L, Richart T, D'hooge J, Thijs L, Fagard RH, Herregods MC, Staessen JA. Left ventricular strain and strain rate in a general population. European Heart Journal, 2008, 29(16):2014–2023.

Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y, Cohen S. Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium? Circulation, 2000, 102(19 Suppl 3):III56-61.

Li RK, Jia ZQ, Weisel RD, Mickle DA, Choi A, Yau TM. Survival and function of bioengineered cardiac grafts. Circulation, 1999, 100(19 Suppl):II63-9.

Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation, 1998,98(25):2800-4.

Lyons E, Pandit A. Design of bioreactors for cardiovascular applications, in: Nureddin Ashammakhi, Rui L Reis (eds). Topics in Tissue Engineering. Volume 2. 2005. Chapter 7.

Lyons F, Partap S, O'Brien FJ. Part 1: scaffolds and surfaces. Technology and Healthcare, 2008, 16(4):305–317.

Mahoney LT, Smith W, Noel MP, Florentine M, Skorton DJ, Collins SM. Measurement of right ventricular volume using cine computer tomography. Investigative Radiology, 1987, 22(6):451–455.

Maidhof R, Tandon N, Lee EJ, Luo J, Duan Y, Yeager K, Konofagou E, Vunjak-Novakovic G. Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue. Journal of Tissue Engineering and Regenerative Medicine, 2012, 6(10):e12–23.

Mann MJ, Dzau VJ. Therapeutic applications of transcription factor decoy oligonucleotides. The Journal of Clinical Investigation, 2000, 106(9): 1071–1075.

Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnology, 2004, 22(2):80–86.

Martin I, Smith T, Wendt D. Bioreactor-based roadmap for the translation of tissue engineering strategies into clinical products. Trends Biotechnology, 2009, 27(9):495–502.

Massai D, Cerino G, Gallo D, Pennella F, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and vascular disease. Journal of Healthcare Engineering, 2013, 4(3):329-70.

McDevitt TC, Angello JC, Whitney ML, Reinecke H, Hauschka SD, Murry CE, Stayton PS. In vitro generation of differentiated cardiac myofibers on micropatterned laminin surfaces. Journal of Biomedical Materials Research, 2002, 60(3):472-9.

McDevitt TC, Woodhouse KA, Hauschka SD, Murry CE, Stayton PS. Spatially organized layers of cardiomyocytes on biodegradable polyurethane films for myocardial repair. Journal of Biomedical Materials Research Part A, 2003, 66(3):586-95.

Melo LG, Pachori AS, Kong D, Gnecchi M, Wang K, Pratt RE, Dzau VJ. Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium: reasons for cautious optimism. Circulation, 2004, 109(20):2386-93.

Mertsching H, Hansmann J. Bioreactor technology in cardiovascular tissue engineering. Advances in Biochemical Engineering Biotechnology, 2009, 112:29–37.

Miao W, Luo Z, Kitsis RN, Walsh K. Intracoronary, adenovirus-mediated Akt gene transfer in heart limits infarct size following ischemia-reperfusion injury in vivo. Journal of Molecular and Cellular Cardiology, 2000, 32(12):2397-402.

Mulieri LA, Hasenfuss G, Leavitt B, Allen PD, Alpert NR. Altered myocardial force-frequency relation in human heart failure. Circulation, 1992, 85:1743–1750.

Müller-Ehmsen J, Whittaker P, Kloner RA, Dow JS, Sakoda T, Long TI, Laird PW, Kedes L. Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. Journal of Molecular and Cellular Cardiology, 2002a, 34(2):107-16.

Müller-Ehmsen J, Peterson KL, Kedes L, Whittaker P, Dow JS, Long TI, Laird PW, Kloner RA. Rebuilding a damaged heart: long-term survival of transplanted neonatal rat cardiomyocytes after myocardial infarction and effect on cardiac function. Circulation, 2002b, 105(14):1720-6.

Nag AC. Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. Cytobios, 1980, 28:41–61.

Nasseri BA, Pomerantseva I, Kaazempur-Mofrad MR, Sutherland FW, Perry T, Ochoa E, Thompson CA, Mayer JE Jr, Oesterle SN, Vacanti JP. Dynamic rotational seeding and cell culture system for vascular tube formation. Tissue Engineering, 2003, 9(2):291–299.

Nuccitelli R. Endogenous ionic currents and DC electric fields in multicellular animal tissues. Bioelectromagnetics, 1992, Suppl 1:147–157.

Odedra D, Chiu L, Reis L, Rask F, Chiang K, Radisic M. Cardiac Tissue Engineering, in: Burdick JA, Mauck RL (eds). Biomaterials for Tissue Engineering Applications. A Review of the Past and Future Trends. 2011. Chapter 15.

Olmer R, Lange A, Selzer S, Kasper C, Haverich A, Martin U, Zweigerdt R. Suspension culture ofhuman pluripotent stem cells in controlled, stirred bioreactors. Tissue Engineering Part C Methods, 2012, 18(10):772–784.

Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Protocol of the National Academy of Science of the United States of America, 2001, 98(18):10344–10349.

Papadaki M, Bursac N, Langer R, Merok J, Vunjak-Novakovic G, Freed LE. Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies. American Journal of Physiology Heart and Circulatory Physiology, 2001, 280(1):168–178.

Pasumarthi KB, Field LJ. Cardiomyocyte cell cycle regulation. Circulation Research, 2002, 90(10):1044–1054.

Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation, 1990, 81:1161–72.

Pok S, Jacot JG. Biomaterials advances in patches for congenital heart defect repair. Journal of Cardiovascular Translational Research, 2011, 4(5):646-54.

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN, Sadek HA. Transient regenerative potential of the neonatal mouse heart. Science, 2011, 331(6020):1078–1080.

Pörtner R, Nagel-Heyer S, Goepfert C, Adamietz P, Meenen NM. Bioreactor design for tissue engineering. Journal for Bioscience and Bioengineering, 2005, 100(3):235–245.

Ptaszek LM, Mansour M, Ruskin JN, Chien KR. Towards regenerative therapy for cardiac disease. Lancet, 2012, 379(9819):933–942.

Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High-density seeding of myocyte cells for cardiac tissue engineering. Biotechnology and Bioengineering, 2003, 82(4):403–414.

Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. Proceedings of the National Academy of Science of the USA, 2004, 101(52):18129–18134.

Radisic M, Park H, Chen F, Salazar-Lazzaro JE, Wang Y, Dennis R, Langer R, Freed LE, Vunjak-Novakovic G. Biomimetic approach to cardiac tissue engineering: oxygen carriers and channeled scaffolds. Tissue Engineering, 2006, 12(8):2077-91.

Radisic M, Marsano A, Maidhof R, Wang Y, Vunjak-Novakovic G. Cardiac tissue engineering using perfusion bioreactor systems. Nature Protocols, 2008, 3(4):719–738.

Radisic M, Sefton MV. Cardiac Tissue, in: Atala A, Lanza R, Thomson JA, Nerem R (eds). Principles Of Regenerative Medicine Second Edition. Academic Press. 2011. Chapter 48.

Ratcliffe A, Niklason LE. Bioreactors and bioprocessing for tissue engineering. Annals of the NY Academy of Sciences, 2002, 96:210–215.

Reffelmann T, Kloner RA. Cellular cardiomyoplasty--cardiomyocytes, skeletal myoblasts, or stem cells for regenerating myocardium and treatment of heart failure? Cardiovascular Research, 2003, 58(2):358-68.

Roger VL, Go AS. Executive summary: heart disease and stroke statistics-2012 update: a report from the American Heart Association. Circulation, 2012, 125(1):188–197.

Schaaf S, Shibamiya A, Mewe M, Eder A, Stöhr A, Hirt MN, Rau T, Zimmermann WH, Conradi L, Eschenhagen T. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. PLoS One, 2011, 6(10):e26397.

Schneck D. An outline of cardiovascular structure and function, in: Bernhard Palsson, Jeffrey A. Hubbell, Robert Plonsey, Joseph D. Bronzino (eds). Tissue Engineering. CRC Press. 2003. I-1–I-12.

Sen A, Kallos MS, Behie LA. New tissue dissociation protocol for scaled-up production of neural stem cells in suspension bioreactors. Tissue Engineering, 2004, 10(5–6):904–913.

Severs NJ. The cardiac muscle cell. Bioessays, 2000, 22:188–99.

Shachar M, Cohen S. Cardiac tissue engineering, ex-vivo: design principles in biomaterials andbioreactors. Heart Failure Reviews, 2003, 8(3):271–276.

Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M, Okano T. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circulation Research, 2002, 90(3):e40.

Shimizu T, Sekine H, Yang J, Isoi Y, Yamato M, Kikuchi A, Kobayashi E, Okano T. Polysurgery of cell sheet grafts overcomes diffusion limits to produce thick, vascularized myocardial tissues. FASEB Journal, 2006, 20(6):708-10.

Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nature Protocols, 2009, 4(2):232-43.

Stevens KR, Pabon L, Muskheli V, Murry CE. Scaffold-free human cardiac tissue patch created from embryonic stem cells. Tissue Engineering Part A, 2009, 15(6):1211-22.

Tandon N, Marsano A, Cannizzaro C, Voldman J, Vunjak-Novakovic G. Design of electrical stimulation bioreactors for cardiac tissue engineering. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2008, 3594–3597.

Tandon N, Cannizzaro C, Chao PG, Maidhof R, Marsano A, Au HT, Radisic M, Vunjak-Novakovic G. Electrical stimulation systems for cardiac tissue engineering. Nature Protocols, 2009a, 4(2):155–173.

Tandon N, Goh B, Marsano A, Chao PH, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G. Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2009b, 6517–6521.

Tandon N, Marsano A, Maidhof R, Wan L, Park H, Vunjak-Novakovic G. Optimization of electrical stimulation parameters for cardiac tissue engineering. Journal of Tissue Engineering and Regenerative Medicine, 2011, 5(6):115–125.

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation, 2002, 105(1):93-8.

Tulloch NL, Murry CE. Trends in cardiovascular engineering: Organizing the human heart. Trends in Cardiovascular Medicine, 2013, 23(8):282-6

Venugopal JR, Prabhakaran MP, Mukherjee S, Ravichandran R, Dan K, Ramakrishna S. Biomaterial strategies for alleviation of myocardial infarction. Journal of the Royal Society Interface, 2012, 9(66):1-19.

Vunjak-Novakovic G, Tandon N, Godier A, Maidhof R, Marsano A, Martens TP, Radisic M. Challenges in cardiac tissue engineering. Tissue Engineering Part B Review, 2010, 16(2):169–187.

Wang Y, Ameer GA, Sheppard BJ, Langer R. A tough biodegradable elastomer. Nature Biotechnology, 2002, 20(6):602-6.

Weinhaus AJ, Roberts KP. Anatomy of the Human Heart, in: Iazzo PA (ed). Handbook of Cardiac Anatomy, Physiology, and Devices. 2nd Edition. 2009. Chapter 5.

Wendt D, Riboldi SA, Cioffi M, Martin I. Potential and bottlenecks of bioreactors in 3D cell culture and tissue manufacturing. Advanced Materials, 2009, 21(32–33):3352–3367.

Wikipedia. http://en.wikipedia.org/wiki/Myocardial_infarction. Accessed December, 2013.

Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. Journal of Molecular and Cellular Cardiology, 2001, 33(5):907-21.

Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnology and Bioengineering, 2000, 68(1):106–114.

Zimmermann WH, Schneiderbanger K, Schubert P, Didié M, Münzel F, Heubach JF, Kostin S, Neuhuber WL, Eschenhagen T. Tissue engineering of a differentiated cardiac muscle construct. Circulation Research, 2002a, 90(2):223–230.

Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T. Cardiac grafting of engineered heart tissue in syngenic rats. Circulation, 2002b, 106(12 Suppl 1):151–157.

Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nature Medicine, 2006, 12(4):452–458.

Zong X, Bien H, Chung CY, Yin L, Fang D, Hsiao BS, Chu B, Entcheva E. Electrospun fine-textured scaffolds for heart tissue constructs. Biomaterials, 2005, 26(26):5330-8.

# PART I

# CHAPTER II

## An electro-mechanical bioreactor for providing physiological-like cardiac stimuli

**Abstract**

The role of physiological-like physical stimuli in improving functional and mechanical properties of the engineered cardiac constructs has been widely demonstrated, and bioreactors can significantly contribute to this objective providing a suitable environment for the development and maturation of engineered cardiac tissues. Within this scenario, an electro-mechanical bioreactor designed for generating a physical environment that mimics the native stimuli of the cardiac tissue has been optimized and implemented. The bioreactor allows to house four cell-seeded cardiac patches and to provide them physiological-like uniaxial cyclic stretching and electrical stimulations. The device has been conceived to be easy of handle, and customizable to match the phenotype of the cultured cells and the maturation phase of the constructs. In-house behavior/operating tests confirmed the suitability and the performances of the bioreactor, demonstrating the fittingness of chamber isolation, grasping system, and motor running. Preliminary cellular tests demonstrated the maintenance of sterility and cell viability, and showed the influence of physiological-like physical stimuli on cardiac constructs. This system is compact and easily fits into a standard cell incubator, representing a highly isolated dynamic cell culture setting suited for long-term experiments. Moreover, the high versatility of the mechanical and electrical stimulation systems allows to use the bioreactor for culturing different types of biological constructs by delivering native-like physical stimuli.

**Keywords:** cardiac tissue engineering, bioreactor design, physiological stimuli, dynamic culture, electrical stimulation, mechanical stimulation.

## 1. Introduction

The innovative filed of cardiac tissue engineering (TE) could represent an effective alternative to overcome the limitations of the current clinical therapies for heart defects. Existing therapeutic cardiac strategies, e.g., transplantation of autologous and donor grafts, and implantation of artificial prostheses or devices as left ventricular assist devices, in fact, suffer from disadvantages. The principal contraindications in using autologous grafts are identified in donor site morbidity, limited availability, risk of infection, and secondary surgical wounds. In case of donor grafts, the disadvantages are the shortage of available donors, the risk of pathogen transfer and rejection, and mandatory lifelong immunosuppressive therapies. Finally, the use of artificial prostheses or devices implies limited durability, inability to completely restore natural functions, and often leads to the establishment of unphysiological conditions with the need of lifelong anticoagulation therapies.

Cardiac TE has shown the potential to generate thick-contractile myocardium-like constructs that might be used as functional substitutes or as biological *in vitro* model systems (Bursac et al., 1999; Habeler et al, 2009; Smits et al., 2009; Hirt et al., 2012) to investigate the cardiac tissue-specific development and diseases, and to offer accurate and controlled *in vitro* tests for cell and cardiac tissue-based therapies (Tulloch and Murry, 2013), drug screening, predictive toxicology and target validation (Elliot and Yuan, 2010; Hansen et al., 2010).

Due to the structural and functional complexity of cardiac tissue, successful strategies for *in vitro* generation of three-dimensional (3D) organized cardiac substitutes imply the use of biomaterials, that should present native-like tissue mechanical properties and/or topographical cues (Kim et al., 2010), as well as there is the need for in-depth investigations on cardiac tissue developmental aspects and adequate biochemical, mechanical and/or electrical stimulations (Ogawa et al., 2007; Figallo, 2008). In this scenario bioreactors, designed for providing native-like culture environments for the development of 3D biological and biochemical processes under monitored, tightly controlled and automated environmental and operating conditions (Martin et al., 2004; Plunkett and O'Brien, 2010), have become essential tools in cardiac research. In fact, it has been demonstrated that, by imposing physiological-like stimulations, it is possible to improve cell differentiation, and structural and functional properties of the construct (Eschenhagen et al., 1997; 2002a; 2002b; Fink et al., 2000; Zimmermann et al., 2002a; 2002b; Gonen-Wadmany et al., 2004; Birla et al., 2007; Kensah et al., 2011). In particular, physiological cyclic stretching increases matrix and cell density, improves morphological tissue organization and interconnection of cells, and promotes contractile characteristics of native myocardium in engineered cardiac constructs (Fink et al., 2000; Zimmermann et al., 2002b; Gonen-Wadmany et al., 2004; Birla et al., 2007). The imposition of auxotonic loads (simultaneous

changes in stress and length), in combination with elevated oxygen supply and supplementation of culture medium with insulin, allows to obtain synchronously contracting multiloop engineered cardiac constructs ready for *in vivo* engraftment (Zimmermann et al., 2006). Moreover, the application of growing static stretch protocols for mimicking the increasing systolic and diastolic pressure in the developing embryonic heart supports sarcomere alignment and cardiomyocyte coupling (Kensah et al., 2013).

By applying physiological electrical field stimuli, ultra-structural differentiation and morphological and constitutive hallmarks of maturing cardiomyocytes can be obtained. In particular, direct current (DC) electric fields, present *in vivo* during embryonic development and wound healing, induce directional cell migration and elongation by modulating morphological and phenotypic characteristics of mesenchymal stem cells, and promote disassembly of gap junctions (Tandon et al., 2009), whereas native-like electrical pulses improve contractility with a marked level of ultra-structural differentiation (Radisic et al., 2004; Tandon et al., 2008; 2009; 2011). Both DC and electrical pulse stimulations upregulate the expression of connexin-43 (Radisic et al., 2004; Tandon et al., 2008; 2009; 2011). The combination of mechanical and electrical stimulations promotes the electrical interconnection through gap junctions of cardiomyocytes, with engineered tissues that present conduction velocity and spatial distribution of connexin-43 similar to the native cardiac tissue (Papadaki et al., 2001; Maidhof et al., 2012) [1].

However, most of these systems are not able to deliver mechanical and electrical stimuli in conjunction, and to allow automated medium recirculation reducing labor work, moreover the set up procedures are often not able to satisfy the good laboratory practice (GLP) rules.

With the aim to overcome the limitations raised above, we have optimized and implemented a previously developed electro-mechanical bioreactor (Mantero et al., 2007) able to generate a biochemical and physical environment suitable for culturing four cell-seeded cardiac patches (Massai et al., 2012; Pisani et al., 2013). In particular, this bioreactor can provide physiological-like uniaxial cyclic stretching (strain 0.5-20%, frequency 1-2 Hz) and/or electrical stimulations (monophasic square pulses, voltage 1-8 V/cm, pulse duration 0-10 ms, frequency 1-10 Hz).

The possibility to adjust the culture parameters to match the phenotype of the cultured cells and the maturation phase of the constructs allows to maximize the efficiency of the regeneration process, and makes this bioreactor a multipurpose adaptable system for dynamic culture of cell-seeded scaffolds for the production of functional engineered constructs.

[1] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

## 2. Materials and Methods

### *2.1 Device requirements*

The principal objective of the electro-mechanical bioreactor is to generate a biophysical environment suitable for growth and differentiation of stem cells cultured on cardiac patches. In detail, the bioreactor can provide accurate and reproducible combined physiological-like physical stimuli, i.e., uniaxial cycling stretching and electrical patterns (Table II.1) according to literature (Mantero et al., 2007; Song et al., 2007; Bouten et al., 2011; Massai et al., 2013).

**Table II.1.** Stimulation parameters for mimicking the native physical stimuli of the cardiac tissue (Mantero et al., 2007; Song et al., 2007; Bouten et al., 2011; Massai et al., 2013).

| Stimulation parameters | Values |
|---|---|
| **Strain (%)** | 0.5 – 20.0 |
| **Frequency of stretching stimulation (Hz)** | 1.0 – 2.0 |
| **Voltage (V/cm)** | 0.1 – 8.0 |
| **Frequency of electrical stimulation (Hz)** | 1.0 – 10.0 |
| **Pulse duration (ms)** | 0.0 – 10.0 |

Moving from these data, the bioreactor was optimized trying to satisfy the following constraints (Mantero et al., 2007):

- cytocompatibility and corrosion-resistance of all the materials in contact with culture medium;
- ease of sterilization and sterility maintenance;
- ease of use (assembly in sterile conditions under a laminar flow hood, cleaning, use for non-trained staff);
- small dimensions, suitable for positioning in a cell culture incubator;
- no medium stagnation during exchange operations;
- housing of an experimentally significant number of specimens.

### *2.2 Architectural design*

The key constitutive elements of the bioreactor are (Figure II.1):

- a transparent, sealable and sterile **culture chamber** where cells, seeded on cardiac patches, will be housed and submerged in culture medium during the entire duration of the experiments;

- a **mechanical stimulation subsystem**, including grasping system, motor and control box, that provides defined uniaxial tensile cyclic loading to the biological constructs and is equipped with a computer-controlled control subsystem;
- an **electrical stimulation subsystem**, including electrodes and voltage source, that provides defined electrical stimulation to the biological constructs;
- a **recirculation subsystem**, composed of a peristaltic pump, oxygen-permeable tubes, a fresh medium reservoir, and a waste receptacle, that is designed to allow automated medium replacement and could be used for maintaining continuous medium recirculation.

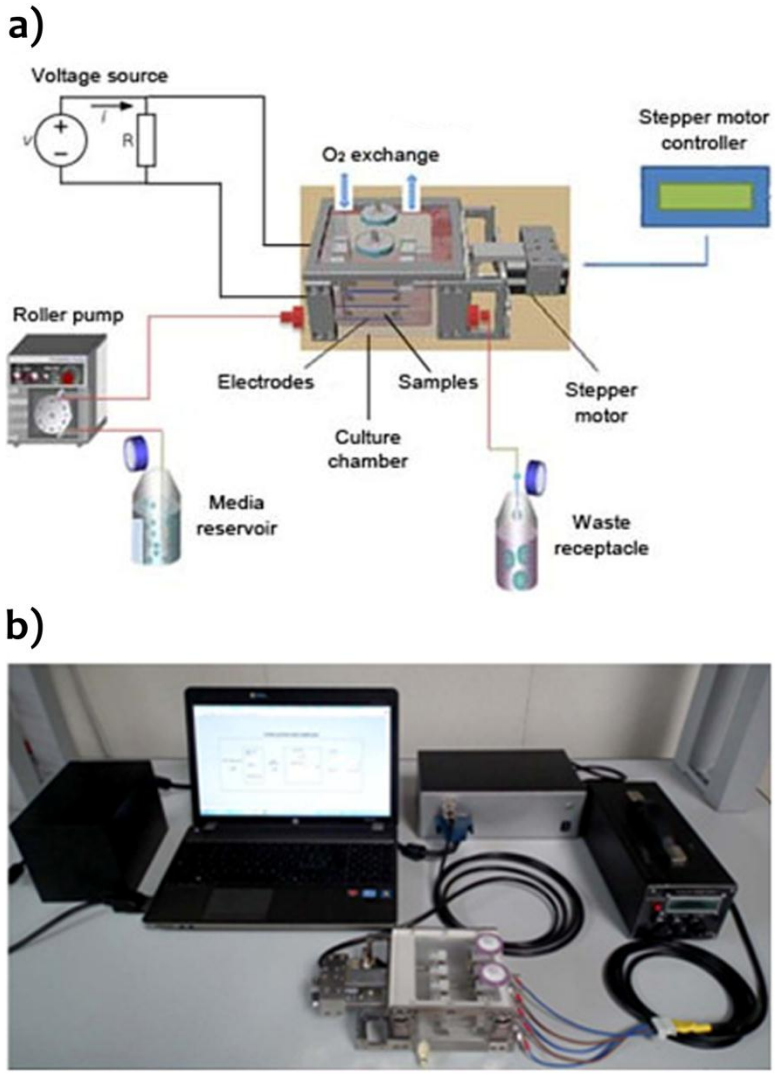


**Figure II.1.** a) Scheme of the bioreactor set-up composed of: bioreactor, recirculation subsystem, mechanical stimulation subsystem, electrical stimulation subsystem. b) Picture of the bioreactor set-up (without the recirculation subsystem).

During the experiment, the bioreactor system is placed within the incubator, with the exception of the controller, the voltage source, and the peristaltic pump that are placed outside to protect them from the humidity (95% - 37°C) that characterizes the internal environment of the incubator (Figure II.2).

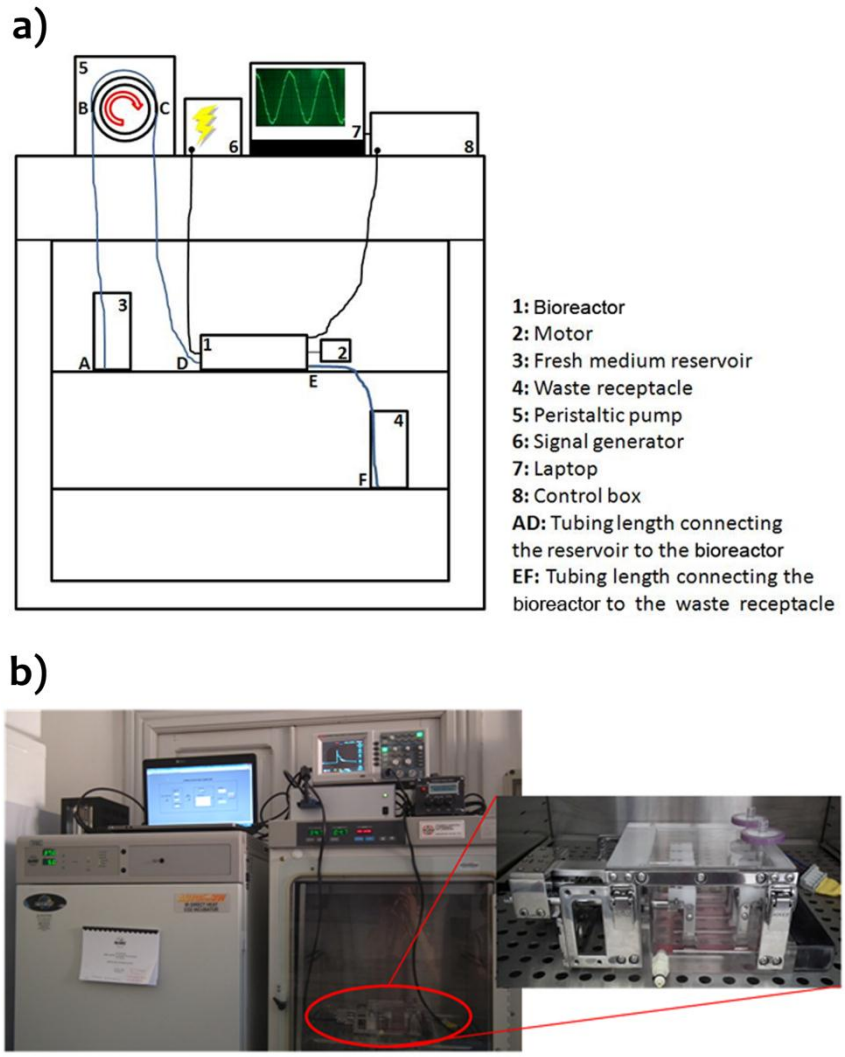


**Figure II.2.** a) Schematic drawing of the bioreactor set-up within the incubator. b) Picture of the bioreactor set-up within the incubator.

## 2.3 Bioreactor constitutive elements

### 2.3.1 Culture chamber

The culture chamber of the bioreactor (component 1 in Figure II.3), i.e., the sterile and cytocompatible environment where cell-seeded cardiac patches are cultured, has been manufactured through material removal by a micrometrical controlled cutter from a Poly(methyl methacrylate) (PMMA) bulk piece, a choice that guarantees the obtainment of transparency. The inner dimensions are approximately 100x97x55 $mm^3$ with a working volume of 70 ml. The rounded edges were conceived in order to avoid stagnation points and discontinuities, fissures, interstices, holes, which are preferable targets for microbial contamination. The chamber is equipped with a lid (component 2 in Figure II.3) that is kept in position by toggle latches (component 8 in Figure II.3), on the top of which two oxygen filters (component 4 in Figure II.3) are located for allowing sterile gas exchange within the incubator. Figure II.3 shows a detailed view of the bioreactor components.

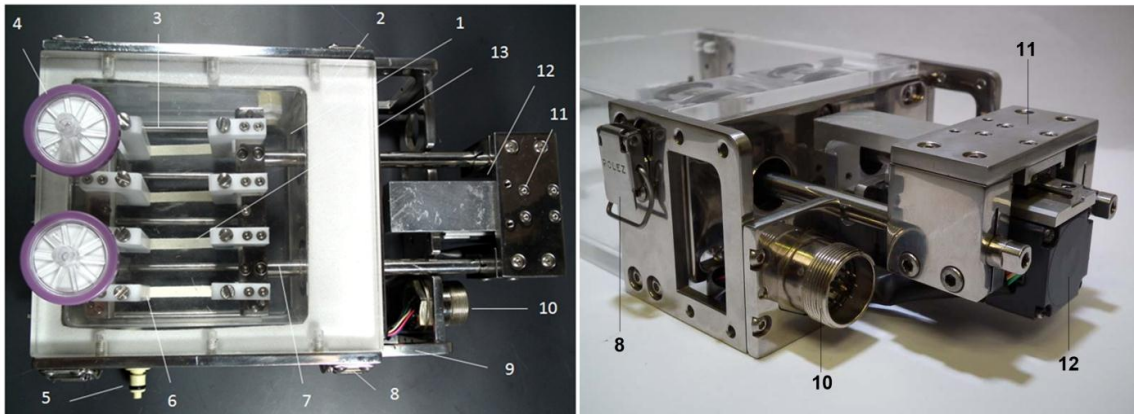


**Figure II.3.** Top and lateral view of the bioreactor components: (1) culture chamber, (2) lid, (3) electrode, (4) filter, (5) quick-disconnected coupling, (6) grip, (7) shaft, (8) latch, (9) frame, (10) cable connector, (11) shaft holder, (12) motor, (13) patch.

### 2.3.2 Mechanical stimulation subsystem

Within the culture chamber, two groups of four grips, each of them screwed onto a crossbar, are positioned symmetrically to hold four constructs (component 6 in Figure II.3). To mechanically stimulate the samples, the mechanical stimulation subsystem is equipped with a micrometrical 5 phase stepper motor (component 12 in Figure II.3) (DRL28PB1G-03, LIMO series, Oriental Motor, Torrance, CA, USA) driving two drive shafts (component 7 in Figure II.3) screwed onto a crossbar that fixes the four mobile grips for the samples. The stepper motor develops a maximum holding force of 30 N, with a 2 μm resolution single step, a maximum acceleration of 0.2 $m/s^2$, and a maximum speed of 24 mm/s. Nominal

stimulation frequency limit is 6 Hz (stepper motor features are reported in Table II.2). A couple of dynamic diaphragms enwraps each drive shaft assuring sterility and allowing motion transmission up to an 8 mm maximum uniaxial dislocation. An AISI 316 rank provides a fastening structure for the stepping motor and for four toggle latches (component 8 in Figure II.3), constituting at the same time two symmetrical handling points.

**Table II.2.** DRL28PB1G-03 stepper motor specifications.

| Parameter | Value (or range) |
|---|---|
| **Number of phases** | 5 |
| **Driving mode** | Half-step |
| **Resolution single step** | 0.72° -4µm |
| **Resolution half step** | 0.36° -2µm |
| **Maximum stroke** | 8 mm |
| **Maximum speed** | 24 m/s |
| **Maximum acceleration** | 0.2 $m/s^2$ |
| **Encoder** | Non present |

In the original version, the grips, manufactured in PMMA, presented a tendency to fracture in a specific region of the base of the grip were the loads were concentrated (Figure II.4).

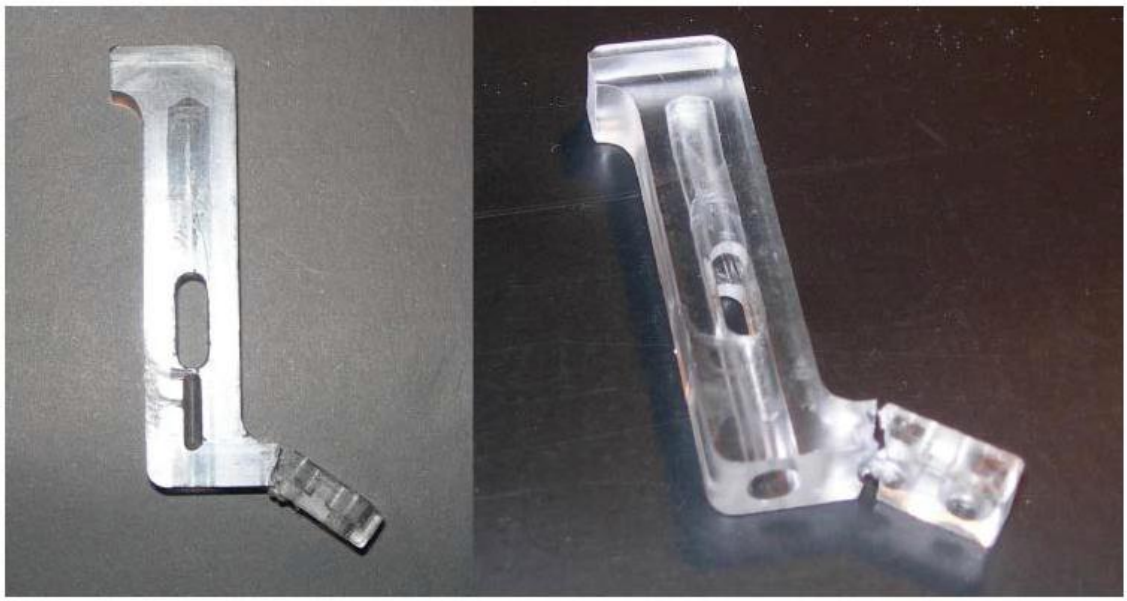

**Figure II.4.** Broken grip.

To get insight into the reason of this unexpected behavior, a finite element method (FEM) model was performed in order to simulate the behavior of the grip under load (*Appendix II.A*).

After the FEM analysis, the grips were re-designed (Figure II.5) in polyoxymethilene (POM) to satisfy both the holding and the preservation of the integrity of the specimen during the experiment, and above all to overcome the problem of fracture and to simplify the method of insertion of the patch.

**Figure II.5.** Bioreactor new grip system.

### *2.3.3 Electrical stimulation subsystem*

The electrical stimulation subsystem is composed of: five AISI 316L electrodes (Figure II.6a), that are placed parallel to the constructs with alternate polarity; and a dedicated external voltage source (Figure II.6b), that provides different voltage-controlled stimulation output signals.

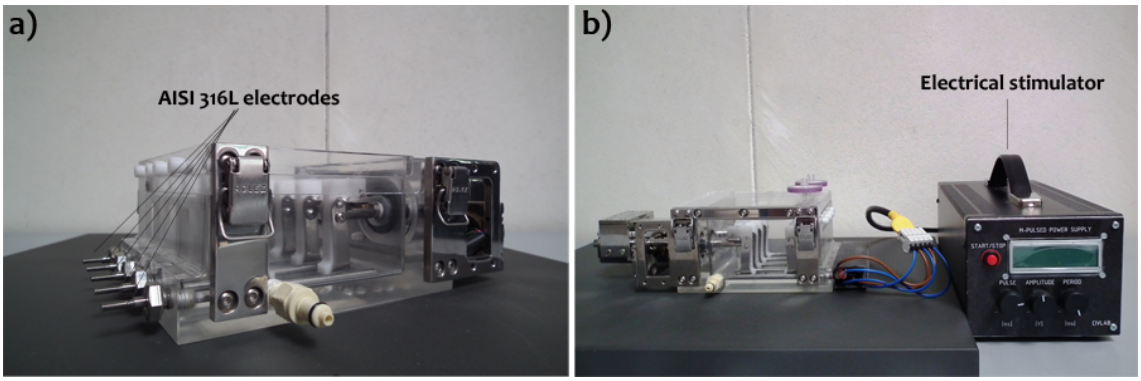


**Figure II.6.** Bioreactor electrical stimulation subsystem. a) Five AISI 316L electrodes. b) Dedicated voltage source.

AISI 316L was chosen to facilitate and reduce the cost of the prototype realization. In the next future carbon electrodes will be used due to their demonstrated high capacitance and high resistance to corrosion, and best charge transfer characteristics (Tandon et al., 2011).

The sealed coupling between the culture chamber and the electrodes is guaranteed by silicone o-rings.

The design of the requirements of the dedicated voltage source is described in *Appendix II.B*.

### *2.3.4 Control subsystem and software*

The schematic diagram reported in Figure II.7 shows the control subsystem components and their mutual connections: the controller, based on a dedicated LabView© platform (Labview© 8.6 version), sends signals to a control card PCI NI MOTION (National Instruments). The latter sends control signals to a driver that provides current signals to the stepping motor.


**Figure II.7.** Schematic diagram of the control subsystem components and their mutual connections.

The control subsystem, placed within the control box, is composed by the circuitry designed to control the stepper motor and the control box tools (Figure II.8).

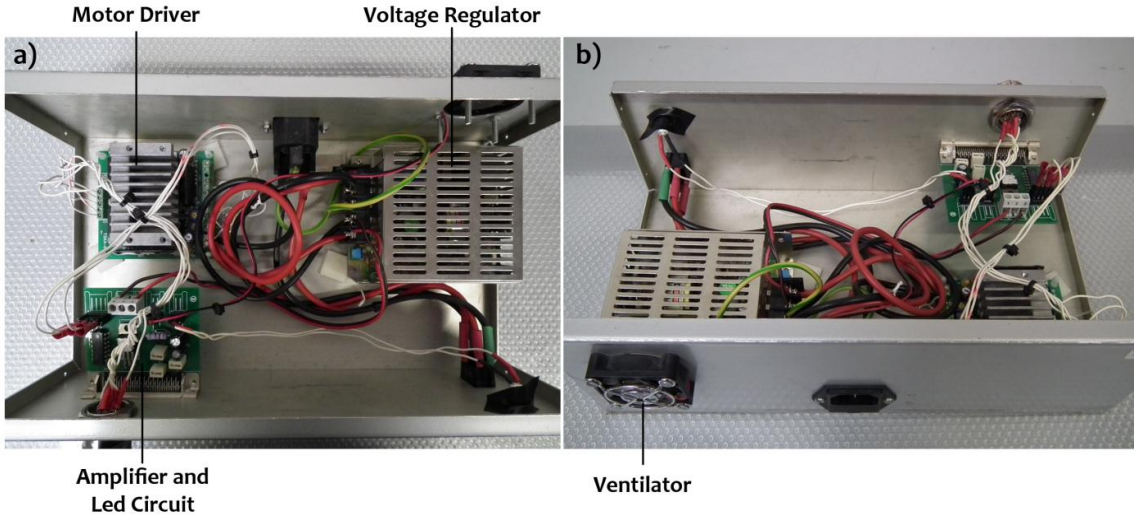


**Figure II.8.** Picture of the inside of the control box.

LabView© platform (Labview© 8.6 version) has been successfully used to realize user-friendly interface and modular software. Firstly, a subunit to control the single axis stepper

motor has been created, and a motion pattern has been generated using the Motion Assistant toolkit (provided by National Instruments). The pattern which has been set is a trapezoidal trajectory, and a subunit dedicated to initialize the stepper motor as the software runs has been added.

The software is composed by four blocks (Figure II.9, top): start, stepper motor initializing, trajectory setting, and motion control block, controlled through a simple control panel (Figure II.9, bottom).

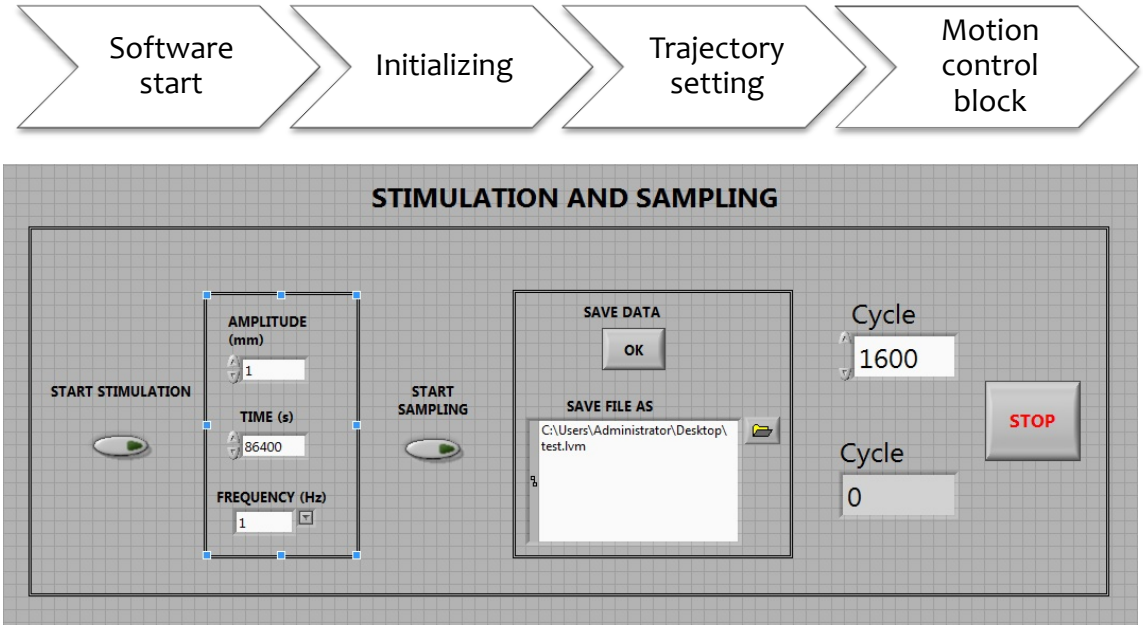


**Figure II.9.** Schematic drawing of software architecture (top). Control panel (bottom).

The first block, named "Software start", allows the user to start operations at any time by clicking on the on/off toggle switch of the control panel. Then, stepper motor is initialized ("stepper motor initializing"). A third block, the "Trajectory setting", is created to configure trajectory parameters (type, acceleration, deceleration, velocity) of the motor unit. This block allows the user to set acceleration, deceleration, and velocity required for the single application. The block named "Motion control block" is responsible for motion pattern control. Within this block, a control has been implemented to halt the execution either by clicking the stop button or by setting the duration of the experiment in the control panel (upper left). These functioning modalities are suitable for a lot of experiments aimed at investigated the environment within the culture cumber during cells stimulation or not.

### *2.3.5 Recirculation subsystem*

The recirculation subsystem has been designed in order to allow automated replacement of the medium and, if necessary, continuous medium recirculation. Two holes were made at

the base of the culture chamber and were equipped with quick disconnected couplings (Figure II.10), guaranteeing the medium exchange through oxygen-permeable tubes and a peristaltic pump.

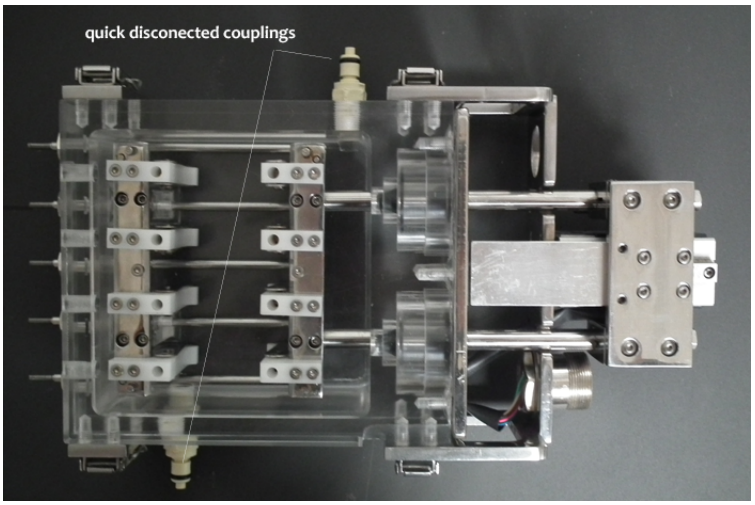


**Figure II.10.** Bioreactor recirculation subsystem set-up.

The recirculation system is composed of: peristaltic pump, oxygen-permeable tubes, fresh medium reservoir, and waste receptacle. The detailed description of the recirculation subsystem is given below.

*Media reservoir*

The role played by the reservoir is dual: it contains sterile media for cell feeding, and it enhances mass transfer by providing oxygenated media (it is a free-surface reservoir).

*Pump*

A peristaltic pump has been chosen (Masterflex L/S® RX-07551 – 00) to assure recirculation. Pump features are summarized in Table II.3:

**Table II.3.** Technical features of the Masterflex L/S® RX-07551 – 00 pump.

| | |
|---|---|
| **Flow rate values** | 0.006 - 3400 ml/min |
| **VAC** | 90/260 |
| **rpm** | 0.1 - 600 |
| **Speed control** | digital precision ± 0.1% |
| **Operating temperature** | 0 - 40°C |
| **Drive dimensions** | 25.4 cm x 21.6 cm x 21.6 cm |
| **Motor** | 75 watts, 1/10 hp |

*Couplings*

Cole-Parmer quick-disconnect couplings (Figure II.11) able to process the fluidic circulation in the system and to ensure unidirectionality of the flow have been chosen. Coupling features are summarized in Table II.4.

**Table II.4.** Technical features of the Cole-Parmer quick-disconnect couplings.

| | |
|---|---|
| **Body material** | Acetal |
| **Seal material** | Ethylene Propylene Rubber (EPR) |
| **Spring and latch material** | 316 Stainless Steel |
| **Max vacuum** | 28" Hg |
| **Max temperarure** | 71°C |
| **Max pressure at 21°C** | 100 psi |

**Figure II.11.** Cole-Parmer quick-disconnected couplings.

*Tubing*

The oxygenation and the hydraulic compliance are assured by the Masterflex BioPharm Platinum-Cured Silicone Pump Tubing (Hose barb 1/8", ID 3.2 mm), retaining the following features shown in Table II.5:

**Table II.5.** Technical features of the Masterflex BioPharm Platinum-Cured Silicone Pump Tubing.

| | |
|---|---|
| **Material** | Tygon 3350 silicone, platinum-cured |
| **Operating temperature** | -60 to 232°C |
| **Methods of sterilization** | Ethylene oxide, gamma irradiation, or autoclave for 30 min, 15 psi |

Permeability properties to $CO_2$, $H_2$, $O_2$ and $N_2$ are summarized in Table II.6.

**Table II.6.** Permeability properties of Masterflex BioPharm Platinum-Cured Silicon Pump Tubing.

| Permeability (approx) at 25°C | | | |
|---|---|---|---|
| $\boldsymbol{Units}: \left\{\frac{cc - mm}{\sec - cm^2 - cmhg}\right\} x\ 10^{-10}$ | | | |
| $CO_2$ | $H_2$ | $O_2$ | $N_2$ |
| 20,132 | 6579 | 7961 | 2763 |

*Waste receptacle*

It is a container for temporarily storing culture wastes.

### *2.4 Tests*

#### *2.4.1 In-house factory tests*

Preliminary in house/behavior tests were conducted to investigate suitability and performances of the bioreactor, by imposing mechanical stimulation only. In particular, tests to assess the fittingness of the chamber isolation, the grasping system, and the motor running were performed. As preliminary tests, KB-C 2000 polyurethane (PU) patches (developed by the Group of Professor Ciardelli, Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Italy), and polyglicerolsebacate (PGS) (developed by the Group of Professor Boccacini, University of Erlangen-Nuremberg, Germany) were used. In detail, the patches were seized in the grasping system with a usable length of 30 mm, housed within the bioreactor, submerged in 70 ml of Phosphate Buffered Saline (PBS) solution at room temperature, and tested imposing uniaxial tensile cyclic loading for five days, with a frequency of 1 Hz and an elongation of the usable length (30 mm) of 15% for the PU patches, and 10% for the PGS patches.

#### *2.4.2 Preliminary cellular tests*

Preliminary cellular tests were performed in order to test the suitability of the device by imposing mechanical stimulation only. In detail, Enhanced Green Fluorescent Protein Cardiac Progenitor Cells ($EGFP^{pos}$ CPCs) isolated from adult EGFP rat hearts (for detailed procedure see Beltrami et al., 2003) and stained with cytoplasmic dye CM-DiI (Invitrogen, UK) were seeded on dense PGS scaffolds functionalized for fibronectin (-GRGDSP) and for laminin (–YIGSR) at the density of $4*10^4$ cells/$cm^2$. PGS scaffold without functionalization was used as control. After seeding, cells were statically cultured for 48 h in complete growth medium (IMDM (Sigma, Italy), 1% P/S (Sigma, Italy), 1% I/T/S (Sigma, Italy), 10% FBS

(Sigma, Italy)) to allow the adhesion of the cells. After 48 h, the samples, four identical strips 40*5*1/2 $mm^3$ in dimension, were subsequently cultured further for 24 h experiencing a stretching pattern (deformation 5%, frequency 1 Hz), and then fixed with paraformaldehyde (PFA) 4% for 1 h at room temperature to allow:

- analysis by fluorescence microscope (Olympus BX60) in order to document the presence and the distribution of $DiI^{pos}$ $EGFP^{pos}$ CPCs on biomimetic patches after mechanical stimulation;
- analysis by a QICAM camera (QImaging, USA) in order to take photomicrographs , then analyzed by a software for image analysis (Image Pro-plus 4.0, Media Cybernetics, Inc., USA) to quantify cell persistence, cell adhesion and cell distribution on patches.

To quantitatively analyze the cell adhesion, the fractional area occupied by red fluorescence and its intensity, expressed as Integrated Optical density (IOD), were then evaluated using a software for image analysis (Image Pro Plus 4.0). All images were acquired with pre-calibrated gain and exposure time. Aspecific fluorescence was carried out by merging the emission signals from different excitation lengths on the same microscopic field. Due to the intrinsic limitations of cell detection on thick membranes, in some experiments, $DiI^{pos}$ $EGFP^{pos}$ CPCs were utilized to visualize the cellular profile under specific fluorescence emission (509 nm) and the presence of DiI within their cytoplasm was assessed.

Cellular tests were performed by the Group of Professor Quaini, Department of Internal Medicine and Biomedical Sciences, Section of Internal Medicine, University of Parma, Italy.

## 3. Results

### *3.1 Tests*

#### *3.1.1 In-house factory tests*

In house behavior/operating tests were performed to demonstrate the suitability and performances of the device.

Stimulating KB-C 2000 PU and PGS patches for five days, imposing uniaxial tensile cyclic loading, with a frequency of 1 Hz and an elongation of 15% or 10% respectively, it has been assessed the fittingness of the chamber isolation, the grasping system, and the motor running.

Both the mechanical and the electrical stimulation can be set up in a simple and easy way, guaranteeing the use by non-experienced staff, and can be applied in conjunction or in an alternating manner, in addition to cell culture medium recirculation. Furthermore, the grasping pressure can be easily regulated by changing the compression through the

internal screw of the grip, allowing a handy insertion and fixation of the patches, and guaranteeing an homogeneous load distribution on the entire patch surface.

### *3.1.2.Preliminary cellular tests*

Preliminary cellular tests demonstrated that all the selected materials are highly cytocompatible and corrosion resistant, and have proved to be appropriate and resistant to the sterilization mode by ethylene-oxide-based process, UV (culture chamber) and by autoclave (grasping system). The set up procedure can be performed under a laminar flow hood, in accordance with the GLP rules, for maintaining the sterility of the culture chamber. Thanks to the limited number of components and to the bioreactor organization, the operations that have to be conducted under laminar flow are just two: (1) insertion of the grasping system within the culture chamber; and (2) grasping of the specimens. Both of them can be easily performed maintaining the sterility of the culture chamber and of the biological constructs during the entire experiment. Moreover, these tests confirmed that, throughout the duration of the experiment, the bioreactor has proved to be a reliable, autonomous and safe device, able to guarantee the sterility and the viability of the cell culture. In particular, the grasping equipment being fully compatible with common laboratory procedures, sample insertion was quickly and easily performed in sterile conditions under a laminar flow hood. The culture chamber, which proved to be extremely comfortable in handling, showed no sign of microbial contamination; air inlets provided the necessary oxygen supply to cultured cells and no release ethylene oxide after sterilization was observed. During stimulation, the device was capable of applying a precise and repeatable predefined stretching pattern to the samples (deformation 5%, frequency 1 Hz), allowing a preliminary investigation of the effect of cyclic mechanical load on cardiac cells. Concerning this aspect, results from preliminary tests demonstrated that $DiI^{pos}$ $EGFP^{pos}$ CPCs adhered both to control and functionalized for fibronectin (-GRGDSP) and for laminin (–YIGSR) patches. In particular, on PGS-GRGDSP membranes, CPCs were distributed following stretching force directions (Figure II.12).

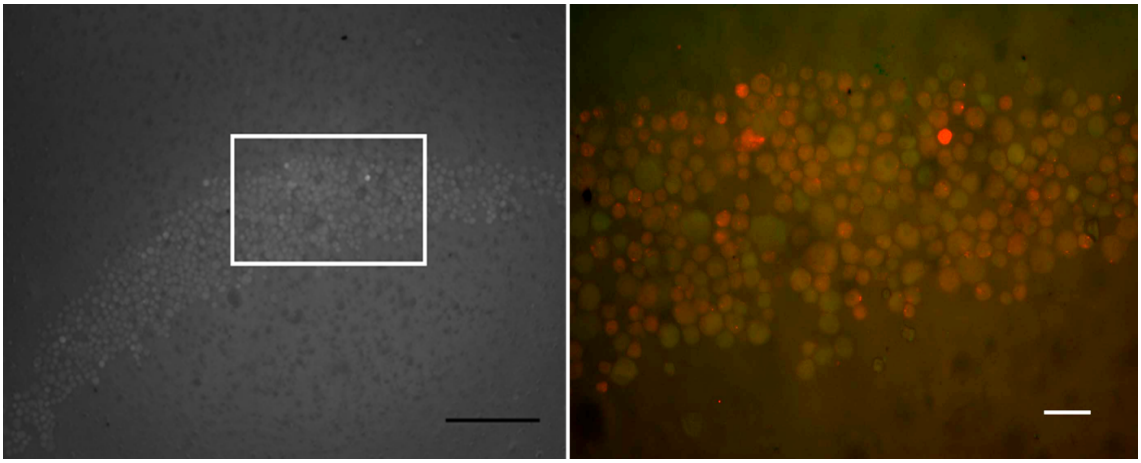

**Figure II.12.** DiI$^{pos}$ EGFP$^{pos}$ CPCs adhered to fibronectin (PGS- GRGDSP)-functionalized patch. Phase contrast microphotograph shows cell distribution following stretching force directions (left). Right microphotograph captured by fluorescence microscope shows CPCs after dynamic culture with CPDCD. Green fluorescence documents GFP and red fluorescence CM-DiI staining (right). Scale bars: 500 µm (left), 100 µm (right).

Moreover, on cardiac patches functionalized with fibronectin, DiI$^{pos}$ EGFP$^{pos}$ CPCs increased in size compared to control PGS (Figure II.13), suggesting a role of mechanical stimulation not only in cell distribution but also in cell volume.

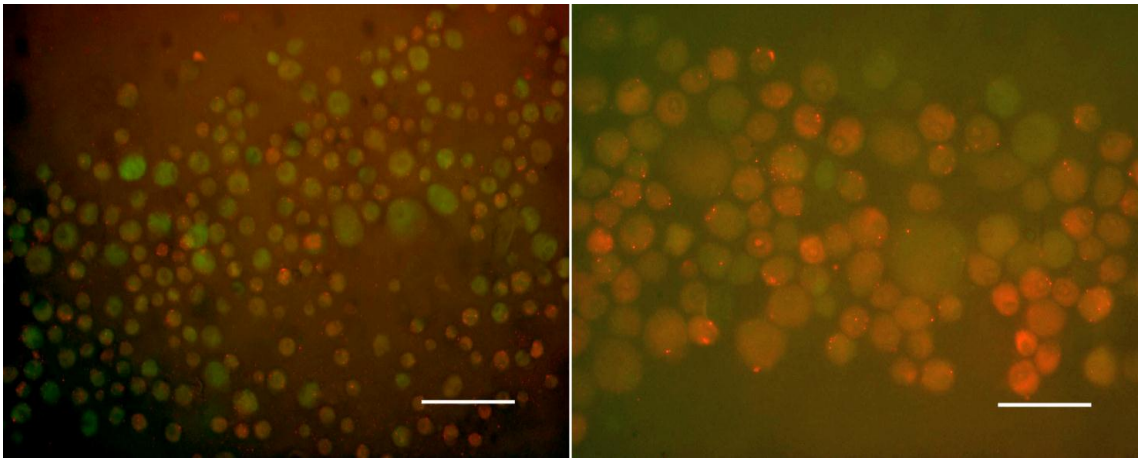

**Figure II.13.** DiI$^{pos}$ EGFP$^{pos}$ CPCs adhered both to PGS-CTRL (left) and GRGDSP functionalized PGS patches (right). Microphotographs were captured at the same magnification after 24 hours of dynamic culture. The increase size of CPCs cultured on PGS- GRGDSP patch is apparent. Scale bars: 100 µm.

Seeding was performed on cells labelled with CM-DiI, a membrane bound red fluorescent dye, which allows the comparative quantification of survival and adhesion to the surface. As expected, compared to standard *in vitro* conditions, the presence of PGS membranes reduced the number of CPCs, but the functionalization seems to improve adhesion and survival (Figure II.14).

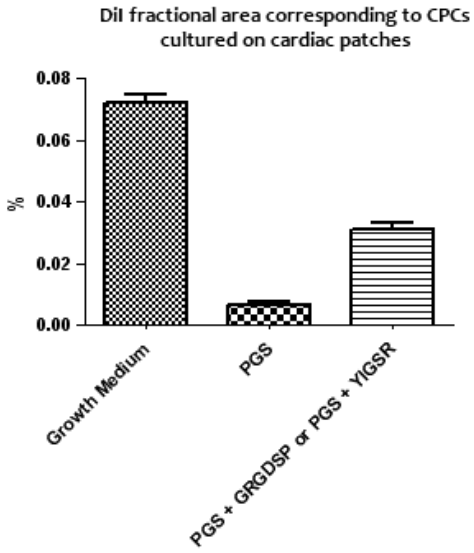


**Figure II.14.** Quantification of the fractional area occupied by DiI labelled CPCs cultured in static condition and considered as control. The fluorescent signal of cells grown in the absence of PGS membranes (Growth Medium) is shown for comparison. Data were grouped for PGS membrane functionalization because either GRGDSP or YIGSR similarly increased the signals of DiI labelled CPCs.

Therefore, finding from preliminary cellular tests demonstrated the potentiality of this bioreactor to be used as a model system for 1) testing cytocompatibility and durability of seeded cardiac patches, and 2) investigating the influence of biophysical stimulation on cells cultured on cardiac patches.

## 4. Discussion and Conclusions

Based on literature data, it is evident that there are increasing efforts in developing *in vitro* strategies and methods for assessing fundamental cardiac biology and physiology. In addition, there is an increasing interest in generation of engineered cardiac constructs *in vitro* to repair damaged tissues *in vivo*. A large number of cell-scaffold-bioreactor systems were designed and used as 3D model systems for investigating the developmental aspects of tissue maturation, and the influence of different biophysical stimuli and mass transport on tissue formation, structure, and function. By ensuring control and monitoring of individual parameters (e.g., cell population, seeding density, physical stimuli) separated from systemic effects existing *in vivo*, these bioreactor-based models provide reliable platforms for (1) assessing the individual and combined effects of each parameter on engineered constructs, and (2) identifying optimal conditions for *in vitro* guiding the generation and maturation of organized and functional cardiac substitutes.

However, most of these systems suffer from some limitations. They are not able to delivery different physical stimuli (mechanical, electrical,) in conjunction, they are not

adaptable to different biological constructs, and, moreover, the set up procedures often do not satisfy the GLP procedures.

The presented electro-mechanical bioreactor was optimized and implemented with the aim to overcome the limitations raised above. This bioreactor is a device able to generate a biochemical and physical environment suitable for conditioning cardiac engineered constructs, by the delivery of uniaxial cyclic stretching and electrical stimulations mimicking the physiological physical stimuli.

Findings of preliminary cellular tests demonstrated the ability of the device to guarantee the sterility and the viability of the cell culture. Moreover, $DiI^{pos}$ $EGFP^{pos}$ CPCs seeded on functionalized PGS patches were observed distributed following stretching direction suggesting a role of mechanical stimulation on cell distribution, and confirming results previously obtained by other research groups (Wang et al., 2004; Pennisi et al., 2011; Delaine-Smith and Reilly, 2012; Salameh et al., 2010).

The grasping system can be easily adapted to the specific biological sample permitting a suitable holding pressure. Once the constructs are housed within the culture chamber, the operator can freely deliver mechanical and electrical stimulation at the same time or in an alternating manner, adding an automated medium recirculation. From the mechanical point of view, it is possible to stimulate cells with cyclic traction and relaxation with tunable amplitudes and frequency, and on the other hand the electrical stimulation has been custom designed for guaranteeing stimulation patterns adapted to different TE applications. Taking together these features, the designed set-up allows to use the device for long-term time-points without any external intervention by the operator.

The possibility to adjust the culture parameters to match the phenotype of the cultured cells and the maturation phase constructs, in order to maximize the efficiency of the regeneration process, makes this bioreactor a multipurpose adaptable system. The design and the implementation of the bioreactor allow to use this device, for cardiac and skeletal muscle TE, due to the electro-mechanical functionalities, and in tendon and ligament TE by using only mechanical functionalities.

Since this bioreactor still suffers from some limitations, further improvements are now ongoing. Significant advantages for a more in-depth understanding of the exact physiological conditions to be reproduced could be achieved by a real-time monitoring and control of both culture operating conditions and construct development. In this context, besides monitoring the milieu parameters (e.g.: temperature, pressure, flow rate, pH, dissolved oxygen and carbon dioxide, metabolite/catabolite concentrations, sterility, etc.), monitoring function and structure of developing engineered cardiac constructs (e.g.: stiffness, force, strength, permeability, composition of the scaffold, cell number, cell viability, etc.) still remains a relatively unexplored area and a highly challenging field of research (Wendt et al., 2009). For example, although contractile function is one of the most important outputs of engineered cardiac tissues, only a few existing platforms are

equipped with systems for quantitative force measurement. Some authors transfer the generated tissue from a cultivation vessel to a measurement device or organ bath chamber (Eschenhagen et al., 1997; 2002b; Fink et al., 2000; Zimmermann et al., 2000; 2002b; 2006; Radisc et al., 2003). A possible weakness of such approach is that force measurement is predominantly used as an end-point analysis, and several samples are needed for long-term data acquisitions, entailing a huge amount of cells needed, more than that for continuous analyses. Recently developed miniaturized platforms attempt to bridge this gap by allowing direct (using specific force sensors (Kensah et al., 2011)) or indirect (using optical analysis (Hansen et al., 2010; Boudou et al., 2012)) measurement of the contraction forces, providing noninvasive online monitoring during prolonged culture[2]. Within this scenario we are now developing a sensing control block responsible for real-time signal acquisition and display without interrupt the cellular experiment. At present, only strain signal is available, but this block has been designed to be modular and ready to be used for pH, temperature, partial pressure of oxygen ($pO_2$) signal acquisition and display. The user-friendly software for both control and acquisition will allow the user to easily perform three types of control over the bioreactor: 1) mechanical stimulation only, 2) real-time sensing only, and 3) mechanical stimulation and real-time sensing. These functioning modalities will be suitable for experiments finalized to investigate the environment within the culture chamber during cell stimulation or not, providing the user with several quantitative data to be used to implement a closed loop control of the experimental parameters. Moreover, in the next future, in order to reduce the number of the control system components and for simplifying the use of the system by non-experienced staff, a single reconfigurable embedded system for the control and data acquisition (NI CompactRIO – National Instruments) will be considered. Finally, specific sensors and control system for the on-line, high throughput monitoring of basic parameters such as temperature, $pO_2$ and partial pressure of carbon dioxide ($pCO_2$) will be implemented.

In conclusion, this bioreactor can be used as a model system for 1) testing cytocompatibility and durability of seeded patches, and 2) investigating the influence of electro-mechanical stimulation on cells cultured on patches has been developed. Moreover, in the near future, the bioreactor can also be used as a production system for implantable functional engineered constructs.

[2] Adapted from: Massai D, Cerino G, Gallo D, Francesco P, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and valcular disease. Journal of Healthcare Engineering. 2013;4(3):329-70.

**Acknowledgements**

Funding for this study was provided by FP7 European Project BIOSCENT. Poly(glycerol sebacate) scaffolds were provided by the Group of Prof. Boccaccini (Institute of Biomaterials, University of Erlangen-Nuremberg, Germany). KB-C 2000 polyurethane scaffolds were provided by the Group of Prof. Ciardelli (Department of Mechanical and Aerospace Engineering, Politecnico di Torino, Italy). The authors would also like to thank Prof. Quaini (Department of Internal Medicine and Biomedical Sciences, Section of Internal Medicine, University of Parma, Italy) for performing cellular tests, and Prof. Civera (CIVLAB, Politecnico di Torino, Italy) for the realization of the voltage source.

## APPENDIX II.A

### *Mechanical stimulation subsystem: FEM model of the grasping system*

In the original version, the grips, manufactured in PMMA, presented a tendency to fracture in a specific region of the base of the grip were the loads were concentrated (Section 2.3.2, Figure II.4). To get insight into the reason of this unexpected behavior, using the properties of PMMA reported in Table II.A1 a FEM model has been created to simulate the behavior of the grip under load. FEM simulation features are reported in Table II.A2.

**Table II.A1.** PMMA properties.

| Feature | Test Method | Units | PMMA XT |
|---|---|---|---|
| Density | DIN 53479 | g/cm3 | 1,19 |
| Tensile strength | DIN 53455 | N/mm2 | 72 |
| Elongation at break | DIN 53455 | % | 4,5 |
| Young's modulus | DIN 53455 | N/mm2 | 3200 |
| Bending strength | DIN 53452 | N/mm2 | 105 |
| Bending modulus | DIN 53452 | N/mm2 | 2900 |
| Brinell hardness H961/30 | DIN 53456 | N/mm2 | 190 |
| Compression strength | DIN 53454 | N/mm2 | 103 |
| Charpy unnotched impact strength | ISO179 -DIN 53453 | kJ/m2 | 12 |
| Charpy notched impact strength | ISO179 -DIN 53453 | kJ/m2 | 2 |
| Water absorption 24h | DIN 53495 | % | 0,3 |

**Table II.A2.** FEM simulation parameters for bioreactor grip analysis.

| | |
|---|---|
| **Number of elements of the mesh** | 708 tetras, 1218 edges, 1664 faces |
| **Method** | Multi-pass adaptive |
| **Polynomial order** | 2 to 7 |
| **Percent convergence** | 25 |
| **Converge on** | Local displacement and local strain |

Constraints have been imposed on the contact point between grip and crossbar, while force is oriented in y-direction and applied on the upper part of the grip (Figure II.A1).

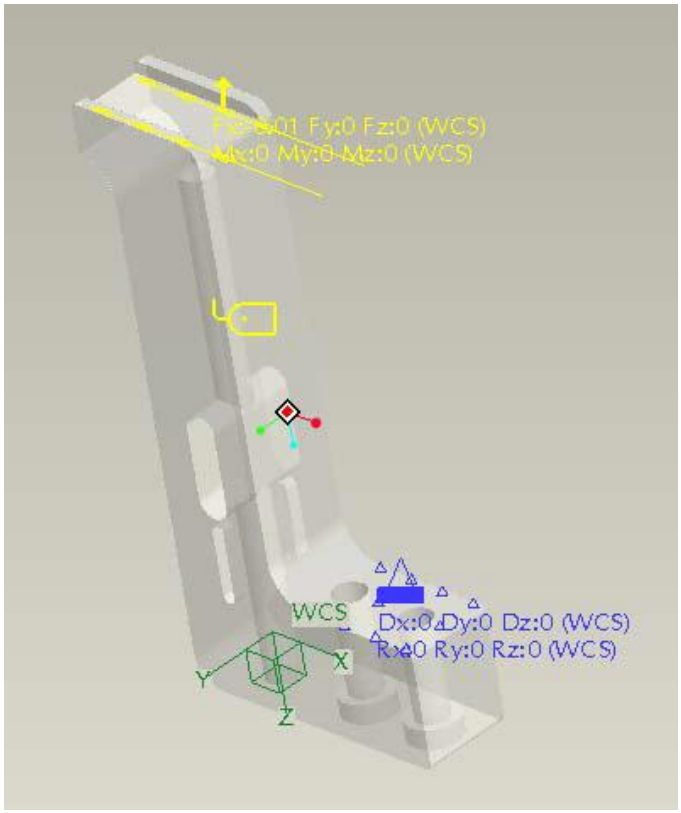


**Figure II.A1.** Constraints and load condition in FEM simulation of bioreactor grip system.

The FEM analysis highlighted the presence of a concentration in the area (that will be named "base of the grip" or simply "base") were the fracture took place (Figure II.A2). The fracture can probably be connected with a fatigue phenomenon.

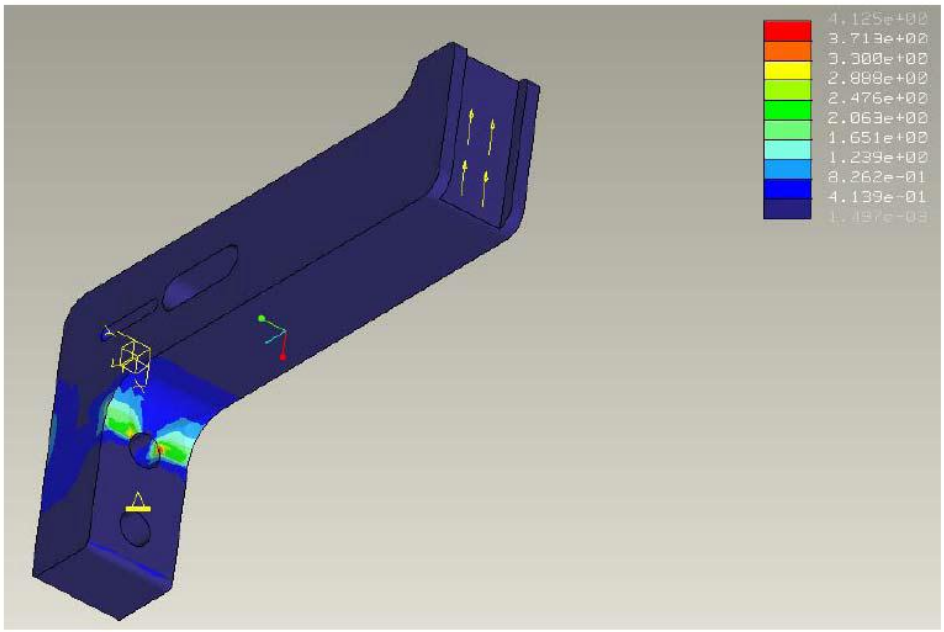


**Figure II.A2.** Von Mises in bioreactor grip system under 1N load.

The idea of redesigning grips was kept under consideration with the aim of adapting the grip geometry to fulfill the potentiality of the system. The stepper motor used in the bioreactor is capable to exert 30 N force as its maximum value. So, the grip should bear a 30 N shear load at its worst condition. It was proved by a FEM simulation that the grip breaks under maximum load because Von Mises at the base covers a range of 60-180 MPa, a quantity that overcomes the tensile strength limit of the PMMA (Figure II.A3).

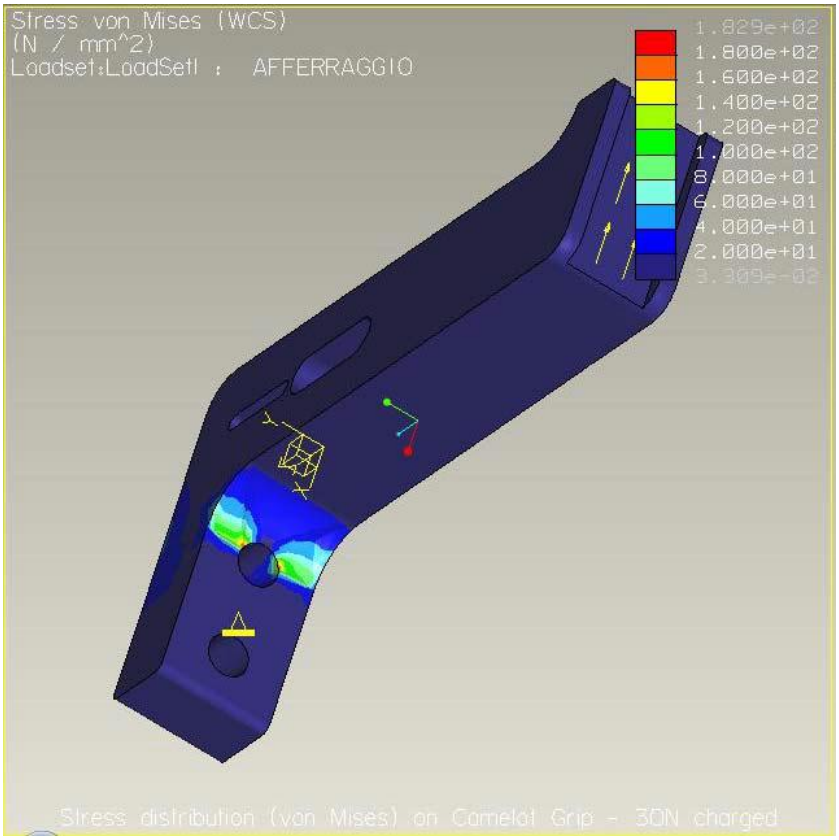


**Figure II.A3.** Von Mises in bioreactor grip system under 30N load (worst case).

For this reason, the grips were re-designed (Figure II.A4) to satisfy both the holding and the preservation of the integrity of the specimen during the experiment, and above all to overcome the problem of fracture and to simplify the method of insertion of the patch.

**Figure II.A4.** Bioreactor new grip system.

The new version for the grip bodies has been obtained from POM, while the internal screw for allowing the patch insertion and fixation, through a compression system, has been manufactured in AISI 316L. Both the body and the screw have been designed to adapt the grasping system to the consistency and thickness dimension of different constructs. POM properties are reported in Table II.A3.

**Table II.A3.** POM properties.

| Quantity | Value |
|---|---|
| **Young's modulus** | 5200-5200 MPa |
| **Shear modulus** | 1500–1500 MPa |
| **Tensile strength** | 62-62 MPa |
| **Elongation** | 12-12 % |
| **Bending strength** | 90-100 MPa |
| **Yield strength** | 62-62 MPa |

## APPENDIX II.B

### *Electrical stimulation subsystem: design of the electrical stimulator*

The implementation of the electrical stimulation subunit has been developed starting from literature data (Tandon et al., 2008). Briefly, 0.004 m diameter rod-shaped electrodes (Figure II.B1A – Φ dimension), 0.08 m in length (Figure II.B1A – L dimension), and placed 0.0235 m between the centers (Figure II.B1B – D dimension), and 0.0195 m between the faces (Figure II.B1B – d dimension), were used.

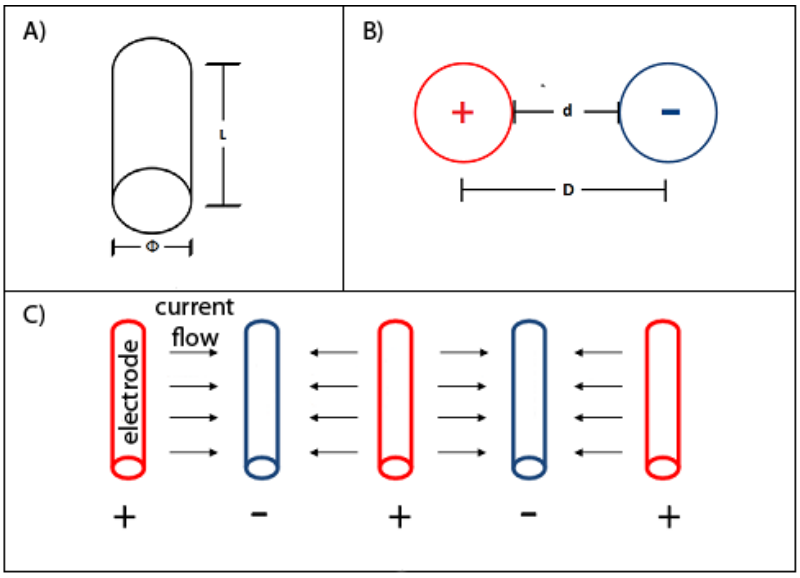


**Figure II.B1.** A) Electrode's dimensions: Φ = 0.004 m diameter rod-shaped electrodes, L= 0.08 m length. B) distances between electrodes with different charge: D = 0.0235 m distance between the centers, d= 0.0195 m distance between the faces. C) Current flow between electrodes.

Medium conductivity value was taken from literature, and it corresponds to 1.5 S/m (Huang et al., 1997). The electrodes were connected in an alternating manner to the electrical stimulator, therefore three electrodes were positively charged while two were negatively charged, in order to ensure the proper current flowing in each construct. Depending on the position and characteristics of the electrode and electrolyte, the electrode-electrolyte configuration was schematized by Randles' Cell (Tandon et al., 2008) represented in Figure II.B2.

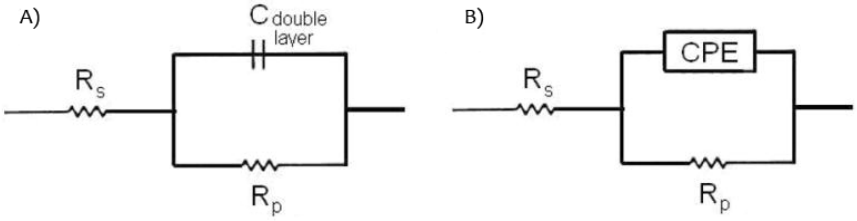


**Figure II.B2.** (A) The equivalent circuit of a simple electrochemical "Randles" cell. (B) The equivalent circuit of a simple electrochemical cell with a CPE instead of a double-layer capacitance (Adapted from Tandon et al., 2008).

In a Randles' cell, Rs represents the resistance of the bulk solution, Rp represents the the electrode's resistance to corrosion, and $C_{double\text{-}layer}$ represents the capacitance of the double layer at the electrode/electrolyte interface. Due to non-homogeneities in electrochemical systems, a so-called "constant-phase element" (CPE) in lieu of a capacitor (Tandon et al., 2008) was used. The resistance of the electrolyte is equal to

$$R_s = \frac{d}{\sigma \cdot A}$$

where A is the surface area exposed to the electrolyte, approximated by rectangle whose length is equal to length electrode and height to half circumference of the electrode.
Therefore,

$A = 0.08 * (0.002*\pi) = 0.0005\ m^2$

accordingly

$Rs = 0.0195 / (1.5*0.0005) = 25.89\ \Omega$

From literature data, the corrosion resistance of the electrode (Rp) is equal to $2.17*10^4\ \Omega/cm^2$, and the value of CPE to stainless steel is $2.30*10^{-5}\ F/cm^2$ (Tandon et al., 2011). With regard to relative quantities to electrical stimulation, in literature is used a typical pulse with stimulation frequency in the range 0-10 Hz, amplitude of stimulation = 2-6 V/cm and a pulse duration of 0-10 ms (Tandon et al., 2008; 2011).

To evaluate the current flowing through the electrolyte, a stimulation program circuit (Pspice version 9.1) was used: the Randles' cell was connected to signal generator (Figure II.B3) which generates square waves with a frequency of 10 Hz, peak to peak voltage of 5V and duration pulse width of 2 ms.

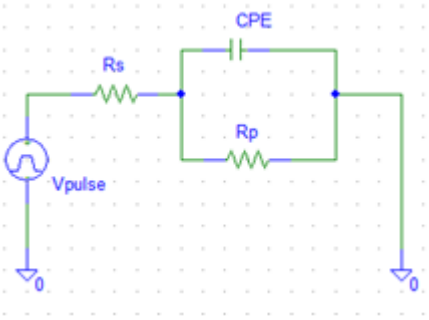


**Figure II.B3.** Circuit simulated with Pspice.

The answer to the current flowing in the electrolyte is represented in Figure II.B4.

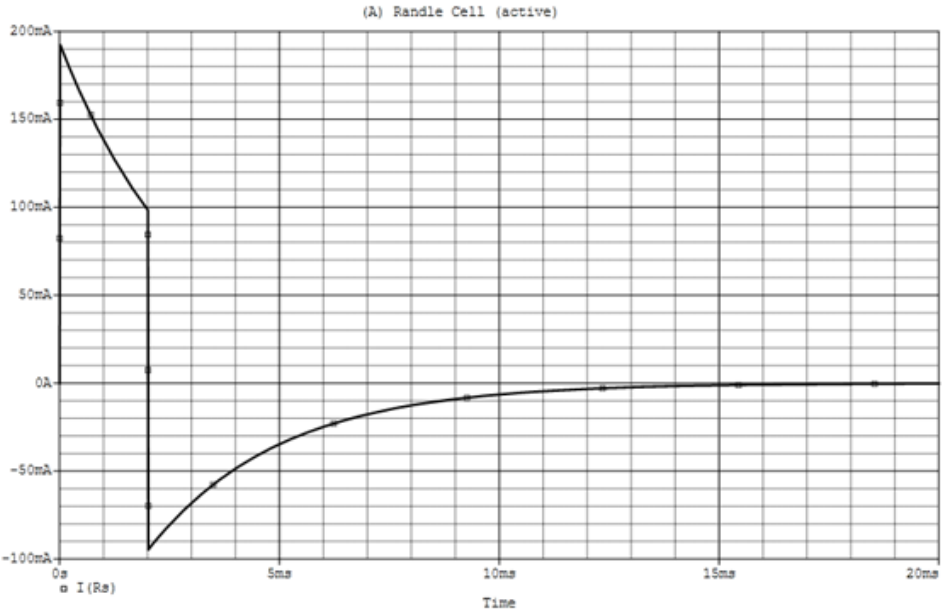


**Figure II.13.** Current flowing through the electrolyte.

Looking at Figure II.B4, it is possible to identify two different behaviors of the current:

1. in the range 0-2 ms, which corresponds to the change in voltage from 0 V to 5 V, the induced current in the circuit goes through the capacitor charging it. Due to the high value of Rp and the capacitor´s charging, the induced current in the circuit assumes a maximum value of Vpp/(Rs||Rp) and decreases exponentially with a constant time τ equal to (Rs||Rp)*CPE (Perfetti, 2013).
2. in the range 2-20 ms, which corresponds to the change in voltage from 5 V to 0 V, the capacitor discharges and the current tends to zero with a time constant τ.

As mentioned, the maximum current value depends on the parallel between Rs and Rp, whose equivalent resistance is equal to 25.89 Ω, almost equal to the Rs value because of the high value of Rp. Therefore, to identify the parameters of the electrical stimulator is possible to consider only Rs.

Taking into account these parameters, the required specifications for the electrical stimulator are:

- peak to peak voltage (amplitude);
- offset;
- maximum current;
- waveform;
- frequency range;
- pulse duration.

Considering the amplitude of stimulation value equal to 6 V/cm (Tandon et al., 2008), the calculation of the potential difference between two points A and B is calculated as (Hayt and Buck, 2006):

$$Vb - Va = \int_{B}^{A} E \cdot dl$$

where Vb is the electrical potential of electrode with positive charge, Va is the potential of the electrode with negative charge, and E is the electric field (6V/cm). Since Va is set equal to zero and the distance between the electrodes is equal to 0.0195 m, Vb is equal to:

$$Vb = E \int_{0}^{d} dl = E \cdot d = 11.7\ V$$

As a consequence, the maximum peak to peak voltage that the electrical stimulator has to supply is equal to 11.7 V, therefore it was decided to apply a maximum voltage of 14V. The offset value is equal to 14 V.

For each pair of electrodes, as mentioned before, the induced current depends on the maximum peak to peak voltage and the resistance Rs, so for the duration pulse of the maximum current is:

I = Vpp/Rs = 452 mA

Since there are four pairs of electrodes, the maximum current that the electrical stimulator has to provide is four times the value needed for a single pair, then equal to 1.81 A, so it was decided to request a maximum current of 1.9 A.

The type of signal that the electrical stimulator has to supply is a pulse wave with a frequency range of 1-10 Hz and a pulse duration in the range of 0-10 ms.

In summary, the electrical stimulator has been designed to satisfy the following required specifications, reported in Table II.B1:

**Table II.B1.** Technical specifications of the dedicated electrical stimulator.

| | |
|---|---|
| **peak to peak voltage** | 0-14V |
| **offset** | 14V |
| **maximum current** | 1.9 A |
| **waveform** | Pulse Wave |
| **frequency range** | 1-10 Hz |
| **pulse duration** | 0-10 ms |

The electrical stimulator was developed by the CIVLAB group of Professor Civera, Politecnico di Torino, Italy.

## References

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell, 2003, 114(6):763-76.

Birla RK, Huang YC, Dennis RG. Development of a novel bioreactor for the mechanical loading of tissue-engineered heart muscle. Tissue Engineering, 2007, 13(9):2239–2248.

Boudou T, Legant WR, Mu A, Borochin MA, Thavandiran N, Radisic M, Zandstra PW, Epstein JA, Margulies KB, Chen CS. A microfabricated platform to measure and manipulate the mechanics of engineered cardiac microtissues. Tissue Engineering Part A, 2012, 18(9–10):910–919.

Bouten CV, Dankers PY, Driessen-Mol A, Pedron S, Brizard AM, Baaijens FP. Substrates for cardiovascular tissue engineering. Advanced Drug Delivery Reviews, 2011, 63(4-5):221-41.

Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. American Journal of Physiology, 1999, 277(2 Pt 2):H433–44.

Delaine-Smith RM, Reilly GC. Mesenchymal stem cell responses to mechanical stimuli. Muscles, Ligaments and Tendons Journal, 2012, 2(3):169-80.

Elliott NT, Yuan F. A review of three-dimensional in vitro tissue models for drug discovery and transport studies. Journal of Pharmaceutical Sciences, 2011, 100(1):59-74.

Eschenhagen T, Fink C, Remmers U, Schols H, Wattchow J, Weil J, Zimmermann W, Dohmen HH, Schäfer H, Bishopric N, Wakatsuki T, Elson EL. Three dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system. Federation of American Societies for Experimental Biology Journal, 1997, 11:683–694.

Eschenhagen T, Didié M, Münzel F, Schubert P, Schneiderbanger K, Zimmermann WH. 3D engineered heart tissue for replacement therapy. Basic Research in Cardiology, 2002a, 97(Suppl 1):146–152.

Eschenhagen T, Didié M, Heubach J, Ravens U, Zimmerman WH. Cardiac tissue engineering. Transplant Immunology, 2002b, 9(2–4):315–321.

Figallo E. Advanced technologies for cardiac tissue engineering. PhD Dissertation, Università degli Studi di Padova, 2008.

Fink C, Ergün S, Kralisch D, Remmers U, Weil J, Eschenhagen T. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. Federation of American Societies for Experimental Biology Journal, 2000, 14(5):669–679.

Gonen-Wadmany M, Gepstein L, Seliktar D. Controlling the cellular organization of tissue engineered cardiac constructs. Annals of the NY Academy of Sciences, 2004, 1015:299–311.

Habeler W, Pouillot S, Plancheron A, Pucéat M, Peschanski M, Monville C. An in vitro beating heart model for long-term assessment of experimental therapeutics. Cardiovascular Research, 2009, 81(2):253-9.

Hansen A, Eder A, Bönstrup M, Flato M, Mewe M, Schaaf S, Aksehirlioglu B, Schwörer A, Uebeler J, Eschenhagen T. Development of a Drug Screening Platform Based on Engineered Heart Tissue. Circulation Research, 2010, 107:35–44.

Hayt W Jr, Buck JA. Engineering Electromagnetics. 7$^{th}$ Edition, 2006. McGraw-Hill Higher Education.

Hirt MN, Sörensen NA, Bartholdt LM, Boeddinghaus J, Schaaf S, Eder A, Vollert I, Stöhr A, Schulze T, Witten A, Stoll M, Hansen A, Eschenhagen T. Increased afterload induces pathological cardiac hypertrophy: a new in vitro model. Basic Research in Cardiology, 2012, 107(6):307.

Huang Y, Wang XB, Becker FF, Gascoyne PR. Introducing dielectrophoresis as a new force field for field-flow fractionation. Biophysical Journal, 1997, 73(2): 1118–1129.

Kensah G, Gruh I, Viering J, Schumann H, Dahlmann J, Meyer H, Skvorc D, Bär A, Akhyari P, Heisterkamp A, Haverich A, Martin U. A novel miniaturized multimodal bioreactor for continuous in situ assessment of bioartificial cardiac tissue during stimulation and maturation. Tissue Engineering Part C Methods, 2011, 17(4):463–473.

Kensah G, Roa Lara A, Dahlmann J, Zweigerdt R, Schwanke K, Hegermann J, Skvorc D, Gawol A, Azizian A, Wagner S, Maier LS, Krause A, Dräger G, Ochs M, Haverich A, Gruh I, Martin U. Murine and human pluripotent stem cell-derived cardiac bodies form contractile myocardial tissue in vitro. European Heart Journal, 2013, 34(15):1134-46.

Kim DH, Lipke EA, Kim P, Cheong R, Thompson S, Delannoy M, Suh KY, Tung L, Levchenko A. Nanoscale cues regulate the structure and function of macroscopic cardiac tissue constructs. Proceedings of the National Academy of Sciences, 2010, 107:565-570.

Maidhof R, Tandon N, Lee EJ, Luo J, Duan Y, Yeager K, Konofagou E, Vunjak-Novakovic G. Biomimetic perfusion and electrical stimulation applied in concert improved the assembly of engineered cardiac tissue. Journal of Tissue Engineering and Regenerative Medicine, 2012, 6(10):e12–23.

Mantero S, Sadr N, Riboldi SA, Lorenzoni S, Montevecchi FM. A new electro-mechanical bioreactor for soft tissue engineering. Journal of Applied Biomaterial and Biomechanics, 2007, 5(2):107-16.

Martin I, Wendt D, Heberer M. The role of bioreactors in tissue engineering. Trends Biotechnology, 2004, 22(2):80–86.

Massai D, Cerino G, Falvo D'Urso Labate G, Rodriguez A, Morbiducci U, Montevecchi FM. Electro-Mechanical Sensorized Bioreactor for Cardiac Tissue Engineering, 3rd TERMIS World Congress 2012, Wien 5-8 September 2012, 6 (Suppl 1).

Massai D, Cerino G, Gallo D, Pennella F, Deriu MA, Rodriguez A, Montevecchi FM, Bignardi C, Audenino A, Morbiducci U. Bioreactors as engineering support to treat cardiac muscle and vascular disease. Journal of Healthcare Engineering, 2013, 4(3):329-70.

Ogawa R, Oki K, Hyakusoku H. Vascular tissue engineering and vascularized 3D tissue regeneration. Regenerative Medicine, 2007, 2(5):831–837.

Papadaki M, Bursac N, Langer R, Merok J, Vunjak-Novakovic G, Freed LE. Tissue engineering of functional cardiac muscle: molecular, structural, and electrophysiological studies. American Journal of Physiology Heart and Circulatory Physiology, 2001, 280(1):168–178.

Pennisi CP, Olesen CG, de Zee M, Rasmussen J, Zachar V. Uniaxial cyclic strain drives assembly and differentiation of skeletal myocytes. Tissue Engineering Part A, 2011, 17(19-20):2543-50.

Perfetti R. Circuiti elettrici. 2nd edition. 2013. Zanichelli, Bologna.

Pisani G, Massai D, Cerino G, Rodriguez A, Audenino A, Morbiducci U. An electro-mechanical bioreactor providing physiological cardiac stimuli, Summer-Workshop on Mechanics in Biology, Berlin 16-17 September 2013.

Plunkett N, O'Brien FJ. Bioreactors in tissue engineering. Studies in Health Technology and Information, 2010, 152:214–230.

Radisic M, Euloth M, Yang L, Langer R, Freed LE, Vunjak-Novakovic G. High-density seeding of myocyte cells for cardiac tissue engineering. Biotechnology and Bioengineering, 2003, 82(4):403–414.

Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. Proceedings of the National Academy of Science of the USA, 2004, 101(52):18129–18134.

Salameh A, Wustmann A, Karl S, Blanke K, Apel D, Rojas-Gomez D, Franke H, Mohr FW, Janousek J, Dhein S. Cyclic mechanical stretch induces cardiomyocyte orientation and polarization of the gap junction protein connexin43. Circulation Research, 2010, 106(10):1592-602.

Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nature Protocols, 2009, 4(2):232-43

Song B, Gu Y, Pu J, Reid B, Zhao Z, Zhao M. Application of direct current electric fields to cells and tissues in vitro and modulation of wound electric field in vivo. Nature Protocols, 2007, 2(6):1479-89.

Tandon N, Marsano A, Cannizzaro C, Voldman J, Vunjak-Novakovic G. Design of electrical stimulation bioreactors for cardiac tissue engineering. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2008, 3594–3597.

Tandon N, Goh B, Marsano A, Chao PH, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G. Alignment and elongation of human adipose-derived stem cells in response to direct-current electrical stimulation. Conference Proceedings - IEEE Engineering in Medicine and Biology Society, 2009, 6517–6521.

Tandon N, Marsano A, Maidhof R, Wan L, Park H, Vunjak-Novakovic G. Optimization of electrical stimulation parameters for cardiac tissue engineering. Journal of Tissue Engineering and Regenerative Medicine, 2011, 5(6):115–125.

Tulloch NL, Murry CE. Trends in cardiovascular engineering: Organizing the human heart. Trends in Cardiovascular Medicine, 2013, 23(8):282-6.

Wang JH, Yang G, Li Z, Shen W. Fibroblast responses to cyclic mechanical stretching depend on cell orientation to the stretching direction. Journal of Biomechanics, 2004, 37(4):573-6.

Wendt D, Riboldi SA, Cioffi M, Martin I. Potential and bottlenecks of bioreactors in 3D cell culture and tissue manufacturing. Advanced Materials, 2009, 21(32–33):3352–3367.

Zimmermann WH, Fink C, Kralisch D, Remmers U, Weil J, Eschenhagen T. Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. Biotechnology and Bioengineering, 2000, 68(1):106–114.

Zimmermann WH, Schneiderbanger K, Schubert P, Didié M, Münzel F, Heubach JF, Kostin S, Neuhuber WL, Eschenhagen T. Tissue engineering of a differentiated cardiac muscle construct. Circulation Research, 2002a, 90(2):223–230.

Zimmermann WH, Didié M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M, Eschenhagen T. Cardiac grafting of engineered heart tissue in syngenic rats. Circulation, 2002b, 106(12 Suppl 1):151–157.

Zimmermann WH, Melnychenko I, Wasmeier G, Didié M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmke H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. Nature Medicine, 2006, 12(4):452–458.