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by

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ABSTRACT

Cardiovascular disease is the leading cause of morbidity and mortality in the Western World. The inability of fully differentiated, load-bearing cardiovascular tissues to *in vivo* regenerate and the limitations of the current treatment therapies greatly motivate the efforts of cardiovascular tissue engineering to become an effective clinical strategy for injured heart and vessels. For the effective production of organized and functional cardiovascular engineered constructs *in vitro*, a suitable dynamic environment is essential, and can be achieved and maintained within bioreactors. Bioreactors are technological devices that, while monitoring and controlling the culture environment and stimulating the construct, attempt to mimic the physiological milieu. In this study, a review of the current state of the art of bioreactor solutions for cardiovascular tissue engineering is presented, with emphasis on bioreactors and biophysical stimuli adopted for investigating the mechanisms influencing cardiovascular tissue development, and for eventually generating suitable cardiovascular tissue replacements.

Keywords: cardiovascular tissue engineering, heart, blood vessels, physiological stimuli, bioreactor

1. INTRODUCTION

Heart disease is the leading cause of morbidity and mortality in the Western World [1, 2], claiming 7.25 million deaths every year, with an increasing trend destined to rise up to about 23.6 million in 2030 [3]. This scenario greatly motivates research into effective therapeutic interventions, since complete native regeneration is unlikely for fully differentiated, load-bearing cardiovascular tissues as myocardium and blood vessels. After myocardial infarction, for example, native cardiomyocytes cannot

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compensate cell loss since they are terminally differentiated cells with no proliferative capacity [4, 5], and the myocardial tissue, poor in muscle stem cells (SCs) [6], lacks significant intrinsic regenerative capability [7]. As a consequence, a marked inflammatory response develops in parallel to a dramatic decrease in nutrients and oxygen supply, leading in short time to scar formation. Scar tissue heavily affects contractility as well as mechanical and electrical properties of native heart muscle, leading to ventricle wall remodeling, and ultimately to heart failure [8, 9]. In patients undergoing end-stage heart failure, heart transplantation becomes a life-saving measure when medical treatments (and less drastic surgical interventions) have failed. However, due to the increasing number of patients and the shortage of available donors, the implantation of left ventricular assist devices (LVADs) has become a clinical reality as an alternative form of treatment [10]. Concerning vascular disease, therapies applied in the clinical practice include the transplantation of native autologous grafts, as the saphenous vein or the internal mammary artery [11, 12], the implantation of artificial grafts [12] or the insertion of stents [13].

However, current therapeutic cardiovascular strategies 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 [12, 14]. 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 [15], and often leads to the establishment of unphysiological conditions with the need of lifelong anticoagulation therapies [12, 16, 17, 18].

Therefore, the innovative field of Tissue Engineering (TE), aiming to completely regenerate three-dimensional (3D) damaged tissues or organs, could represent an effective alternative to overcome the current clinical limitations. Attempts to *in vivo* stimulate the regeneration of injured tissues were pursued on animal models by: (1) injection of differentiated cells or SCs *in situ* [19, 20]; (2) mobilization of endogenous SCs with cytokines [21]; (3) activation of cardiomyocyte cell cycle [22, 23] obtained, e.g., by inducing permanent coronary artery occlusion [24] or performing apical ventricular resection [25, 26]; 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 [27]. 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 [25]. 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 [28]: (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 [29, 30].

Due to the structural and functional complexity of cardiac and vascular tissues, successful strategies for *in vitro* generation of 3D organized cardiovascular substitutes require in-depth investigations on tissue developmental aspects and adequate biochemical, mechanical and/or electrical stimulations [31, 32]. In this scenario, dynamic culture devices (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 [33, 34], have become essential tools in cardiovascular research. In detail, the use of bioreactors allows (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, thus improving the morphological and functional properties of the engineered constructs [27, 35, 36, 37, 38]. Therefore, bioreactors are widely used as model systems to investigate *in vitro* tissue maturation and the effects of mass transport and biophysical and chemical stimuli on tissue formation. Once optimal culture conditions have been identified, bioreactors can be used as production systems for *in vitro* generation of engineered functional tissues. In recent years several studies have shown that the use of bioreactors in industrial processes for TE is sustainable both clinically and economically [39, 40, 41, 42], demonstrating that the use of closed, standardized, and automated systems guarantees more reproducibility and lower contamination risk than production processes carried out manually [43, 44, 45, 46]. Moreover, bioreactors, in combination with recent induced pluripotent SC technology, have been used for producing *in vitro* models of disease [47, 48].

This review aims to provide an up-to-date overview of bioreactors used as engineering support in cardiovascular TE, with particular focus on the use of bioreactors (1) to investigate the still unknown mechanisms of cardiovascular tissue development and the role of specific biophysical stimuli, and (2) to produce cardiovascular engineered tissues to be implanted in animal models. Particular emphasis is given on technological solutions for delivering physical stimuli that mimic the physiological environment. After discussing the key properties and stimuli of native cardiac and vascular tissues, that should be considered in the design of advanced bioreactors for the generation of human engineered cardiovascular substitutes, the state of the art of bioreactors for *in vitro* investigation and generation of cardiac and vascular tissues is reviewed. Furthermore, works are discussed focusing on specific stimulation requirements for effective bioreactors, pointing out future challenges in development of next generation bioreactors for clinical use, and advantages of adopting bioreactors in clinical strategies.

2. METHODS OF SEARCH

To carry out an exhaustive and complete review of the state of the art of bioreactors for cardiovascular TE, two different search methods have been adopted: (1) a PubMed search to identify related papers and books published from 1957 to 2012, using the following keywords: heart disease, cardiovascular TE, bioreactors, heart, blood vessels, cardiac patch, vascular graft, electrical stimulation, mechanical stimulation, perfusion,

physiological stimuli; (2) a constant and thorough analysis of the state of the art, including the most recent knowledge acquired from courses and congresses pertaining to TE research.

3. PROPERTIES AND STIMULI OF CARDIOVASCULAR TISSUES

To investigate and guide *in vitro* cellular growth and differentiation, and functional tissue organization, it is fundamental to design biologically inspired environments that mimic physiological conditions, since cells and tissues correctly grow and mature if they are subjected to physical stimuli similar to the physiological ones [27, 37, 38, 39, 40]. In fact, cells respond to the entire context of their environment (molecules, other cells, matrix, physical forces, etc.) and it has been widely demonstrated that specific 3D dynamic culture conditions enhance size, cellular homogeneity, molecular composition, and functionality of the engineered constructs, in comparison with conventional static cultures on flat surfaces.

Table 1. Properties and stimuli of human cardiovascular tissues

Property/stimulus	Heart	Blood vessels
Internal diameter	Left ventricle: 3.3-7.5 cm [49]	Large arteries: 4-5 mm [50] Capillaries: 3.5-10 μm [50]
Wall thickness	Left ventricle: 8-15 mm [43] Right ventricle: 4-5 mm [43]	Large arteries: 1 mm [50] Capillaries: 0.5-1 μm [50]
Length	–	Large arteries: 1.4-2.8 cm [50] Capillaries: 0.5-1.1 mm [50]
Volume	Left ventricle: 40-130 ml [43,51] Right ventricle: 24-86.5 ml [52]	–
Resting rate	60-100 bpm (1-1.7 Hz) [50,53]	–
Young's Modulus	0.2-0.5 MPa (end diastolic value) [8]	0.2-1.4 MPa [12]
Pressure	Left ventricle: 10-120 mmHg [43] Right ventricle: 5-30 mmHg [50]	Large Arteries: 80-120 mmHg [54,55] Capillaries: 30 mmHg [50]
Burst pressure	–	1600-3200 mmHg [55]
Flow rate	–	Large Arteries: 115 ml/min [54] Capillaries: $5 \cdot 10^{-4}$ ml/min [56]
Shear stress	–	Venous system: 1-6 dyne/cm^2 [57] Arterial system: 10-70 dyne/cm^2 [57]
Mechanical load	50 kPa [43]	–
Strain	Longitudinal: 22.9% [58] Radial: 59.2% [58]	Circumferential: 10-15% [43] Longitudinal: 40-65% [59]
Electrical field	0.1-10 V/cm [60]	–
Electrical pulse duration	1-2 ms [60]	–

bpm: beats per minute

In order to provide design criteria for advanced bioreactors for an effective generation of human implantable functional engineered constructs, that still remains one of the major challenges in cardiovascular TE research, the structural and functional properties of human native heart and blood vessels and the measured physical stimuli acting on them are summarized in Sections 3.1 and 3.2. Moreover, since the majority of the state-of-the-art studies use animal model cells in combination with bioreactor environment to generate engineered constructs to be used as *in vitro* model systems, physiological stimuli typical of the adopted animal models are summarized at the end of each section.

3.1. The Heart

The mammalian heart is a dynamic electromechanical system where the myocardial tissue undergoes mechanical stretch during diastole and active contraction during systole, consuming large amounts of oxygen. The bulk of the heart tissue is the contractile myocardium, a structure with asymmetrical and helical architecture [61, 62], composed of tightly packed rod-shaped myocytes (forming myofibers) and fibroblasts, with dense supporting vasculature and collagen-based ECM. Cardiac myocytes are highly metabolically active; therefore oxygen and nutrients are depleted within a relatively thin layer of viable tissue [63]. Native myocardium obviates this difficulty through a rich vasculature pervading the cardiac muscle.

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 [43]. 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 [43,51], respectively. The right ventricle pressure ranges between 5 to 30 mmHg [50], and the cavity volume changes from 24 to 86.5 ml [52]. Local mechanical loads can reach 50 kPa [43], with 22.9% longitudinal and 59.2% radial mean strain [58]. 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 [64, 65]. End diastolic values for the Young's modulus have been reported to range between 0.2 and 0.5 MPa [8]; 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) [66]. For vertebrates, the physiologically significant range of endogenously produced electrical field strengths is 0.1-10 V/cm [60]. 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 [67]. 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 [50,53]. In terms of pulse duration, 1-2 ms is sufficiently long to excite heart tissue cells [60].

Regarding animal models, the use of rats dominates in cardiac TE research due to their low cost, ease of handling and cell isolation procedures, and ability to maintain rat cardiomyocytes growing and beating for long periods *in vitro* (at least two weeks). Moreover, cell cultures of neonatal rat cardiomyocytes are a well characterized standard model, due to the genetic information available in much greater detail in rat than in many other species [68]. Concerning physiological stimuli present in rat heart, systolic pressure ranges between 100 and 120 mmHg, diastolic pressure ranges between 70 and 80 mmHg, and beat frequency ranges between 5-7 Hz for an intact rat adult heart [69] and 4-6 Hz for a neonatal rat heart [70, 71].

3.2. Blood Vessels

Blood vessels form a branched system of arteries, capillaries and veins with different sizes, biochemical and cellular contents, mechanical properties, and ultra-structural organizations depending on their locations and specific functions [45]. They are composed of different concentric layers: (1) tunica intima, the inner layer, consisting of endothelial cells (ECs) lining the lumen and supported by a connective tissue bed; (2) tunica media, the middle layer, consisting of circumferentially arranged layers of smooth muscle cells (SMCs) alternated with elastin sheets; and (3) tunica adventitia, the outer layer, composed of collagen fibers, that anchors the vessel to its surroundings [43, 45, 72].

In the human body, the size of blood vessels varies enormously. In large arteries, the internal diameter (ID) ranges from 4 to 5 mm and the length from 1.4 to 2.8 cm, while in capillaries, the ID varies from 3.5 to 10 μm, and the length from 0.5 to 1.1 mm [50]. Wall thickness varies from 0.3 to 1 mm, being largest in the large arteries, much less in veins of comparable diameter, and only a single cell thick in the capillaries, i.e., 0.5-1 μm [50]. Coronary arteries typically have a diameter of 3-4 mm and a wall thickness of 1 mm [43].

The major vessels are perfused by pulsatile blood flow and expand each time the heart contracts, and then recoil elastically while the heart is refilling, continuously providing blood to the small peripheral vessels and capillary beds. Blood pressure in large arteries typically varies from 120 (systole) to 80 (diastole) mmHg, with an average flow rate of 115 ml/min [54,55]. In capillaries, the pulsatility is lost and pressure is only about 30 mmHg, with an average flow rate of approximately $5 \cdot 10^{-4}$ ml/min [56].

Blood vessels must be distensible to provide capacitance and pulse-damping in the circulation, but they must also be stable to inflation over a range of pressure [73], presenting a non-linear elastic behavior. The Young's modulus of the vascular wall increases with strain, being about 0.2 MPa at diastolic pressure, and about 1.4 MPa at systolic pressure [12]. The endothelial lumen is constantly exposed to hemodynamic shear stresses that range from 1 to 6 dyne/cm^2 in the venous system, while from 10 to 70 dyne/cm^2 in the arterial vascular network [57]. The burst pressure ranges from 1600 mmHg for the saphenous vein to about 3200 mmHg for the internal mammary artery [55]. Circumferential wall strain ranges between 10% and 15%, whereas longitudinal strain ranges from 40% to 65% [74].

Ovine and bovine species are animal models widely used in vascular TE research due to their large size and similarity with respect to human vascular tissue. Ovine common carotid artery is around 100 mm long, without any bifurcation, and 4 mm in diameter. The ovine normal heart rate ranges from 50 to 80 bpm (0.8 - 1.3 Hz), the maximum heart rate ranges from 260 to 280 bpm (4.3 - 4.6 Hz), and the mean arterial pressure is 70 mmHg [75]. Bovine species present arteries of approximately 5 mm in diameter [76], heart rate ranging from 36 to 84 bpm (0.6 - 1.4 Hz) [77], and arterial mean pressure at heart level ranging from 84 to 227 mmHg, associated respectively with systolic/diastolic pressures of 118/62 and 275/195 mmHg [78]. Although used less often, dogs are also considered a valuable model for studying vascular disease due to their convenient size [79].

4. BIOREACTORS

As already discussed, 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 [27]. 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 [80]. 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* [27, 33, 81]. 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 [45, 46].

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) [43, 82]. Advanced bioreactor systems should be equipped with the following (Figure 1): (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 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 [27, 39]. Therefore, for an effective *in vitro* tissue generation strategy, it is essential to define not only culture actors (cells, scaffolds, culture medium, growth

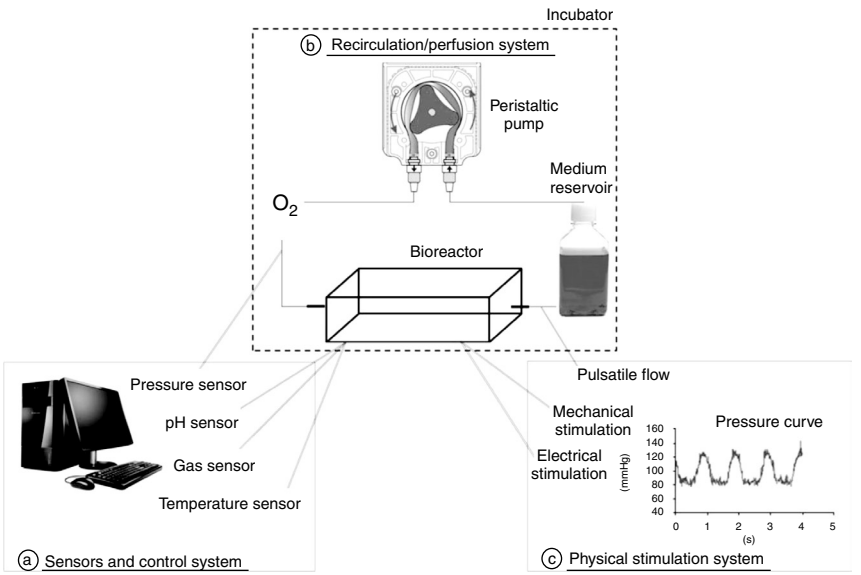

Figure 1. Advanced bioreactor system equipped with (a) sensors and control systems, (b) recirculation/perfusion systems, and (c) physical stimulation systems.

factors, etc.), but also bioreactor's design criteria, which are dictated by the tissue to be produced [27, 83].

Focusing on bioreactors designed for the production of cardiovascular constructs suitable for implantation, the presence of physical stimulation systems (i.e., mechanical stretching, electrical stimulation, pulsatile flow) is fundamental for promoting the modulation of cell behavior in terms of proliferation [84], differentiation [85], protein synthesis and ECM remodeling [86], and for promoting structural and functional tissue maturation in terms of 3D morphology [35], mechanical strength [87] and electrical function [60]. 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 [88]. An overview of the state of the art of bioreactors is provided below for the study of phenomena involved in the mechanism of cardiovascular tissue formation, and for the *in vitro* generation of cardiac and vascular tissues.

4.1. Bioreactors for Cardiac Tissue Engineering

As already discussed, the human heart has a limited capacity to regenerate itself [89]. 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 [9, 69]. The complexity of the cardiac tissue makes the fulfillment of these requirements very challenging, since adult cardiomyocytes 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 [60]. Table 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 cardiovascular research and tissue replacement therapy.

In their pioneering studies [90, 68, 105], Eschenhagen, Zimmermann and co-workers proposed a method for the *in vitro* production of coherently contracting 3D engineered heart tissues (EHTs) made of cardiac myocytes from embryonic chicken [90] and neonatal rats [68, 106] 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 2). EHTs exhibited well-organized myofilaments with intercellular connections, and coherent contractions after 2-3 days [68, 90].

The influence of chronic mechanical stretch on morphological and functional behavior of cardiac myocytes was evaluated by Fink *et al*. [80], who subjected EHTs to phasic unidirectional stretch (1-20%, 1.5 Hz) for 6 days and then to isometric force measurement (as in [68, 90]). Stretched EHTs exhibited improved organization of cardiac myocytes 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 sheet-shaped EHTs, ring-shaped rat EHTs were cast [91]. After 7 days of culture, ring-shaped EHTs were transferred into a modified stretch device (Fig. 2a) and submitted to unidirectional cyclic stretch (10%, 2 Hz) for 7 days; afterward, EHTs were subjected to isometric force measurements (as in [68, 90]). On circular EHTs, Zimmermann *et al*. [91] observed complexes of multicellular aggregates and longitudinally oriented cell bundles, with morphological features of adult tissue.

Table 2. Devices and bioreactors for cardiac tissue engineering

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Eschenhagen *et al.*, 1997 [90]	Embryonic chick cardiac myocytes	Cell/Type I collagen mixture	Organ bath (static flow)	Stepwise adjusted preloads Electrical rectangular pulse (10 ms, 20-40 V, 0.8-2 Hz)	Contractile activity	Resting tension: 1.15-2.81 mN Twitch tension: 0.09-0.2 mN Beating: 72.4 ± 2.6 bpm
Zimmermann *et al.*, 2000 [68]	Neonatal rat cardiac myocytes	Cell/Type I collagen/Matrigel mixture	Organ bath (static flow)	Stepwise adjusted preloads Electrical rectangular pulse (5 ms, 1 Hz)	Contractile activity	Resting tension: 0.20-0.63 mN Twitch tension: 0.21-0.46 mN Beating: 180 bpm
Fink *et al.*, 2000 [80]	Embryonic chick cardiac myocytes	Cell/Type I collagen/Matrigel (rat) mixture	Stretch device (static flow) Organ bath (static flow)	Cyclic unidirectional stretch (1-20%, 1.5 Hz)	Contractile activity	Force of contraction of stretched (20%) EHTs:

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
	Neonatal rat cardiac myocytes			Stepwise adjusted preloads		0.10-0.18 mN (chicken, 238% over controls) 0.20-0.65 mN (rat, 188% over controls)
				Electrical rectangular pulse (10 ms, 20-40 V, 1.5 Hz (chick) or 2 Hz (rat))		Twitch duration: decrease of 14-44% with respect to unstretched controls
Zimmermann *et al*., 2002 [91]	Neonatal rat cardiac myocytes	Cell/Type I collagen/Matri-gel 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 (1 Hz)		Twitch tension: 0.36 (basal condition) -0.75 mN

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Gonen-Wadmany et al., 2004 [87]	Neonatal rat cardiac myocytes 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)	–	–
Birla *et al*., 2007 [92]	Neonatal rat cardiac myocytes	Fibrin gel with thrombin	Mechanical stimulation bioreactor (static flow)	Cyclic stretch (10%, 1 Hz) Parallel electrodes (10 ms, 15 V, 1 Hz) with custom-built optical force transducer	Contractile activity	Mean specific active force: 12.1 ± 0.62 kN/m^2
Akins *et al*., 1999 [93]	Neonatal rat cardiac myocytes	Fibronectin-coated polystyrene beads Oriented collagen fibers	HARV rotating bioreactor (laminar flow) Transparent Teflon culture bags (slowly rotated)	–	Contractile frequency (real time for Teflon culture bags)	–

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Carrier *et al*., 1999 [35]	Neonatal rat cardiac myocytes Embryonic chick cardiac myocytes	Porous, disk-shaped, nonwoven mesh of fibrous PGA	Flasks (static and stirred turbulent flow, 50-90 rpm) Dishes mounted on *xyz* gyrator (mixed flow, 24 rpm) Rotating-wall bioreactor (dynamic laminar flow, 11-12 rpm)	–	Culture parameters (pH, pO_2, pCO_2) Cell damage and death (measuring medium lactate dehydrogenase levels in supernatant)	–
Papadaki *et al*., 2001 [89]	Neonatal rat cardiac myocytes	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)	Electrical pulses (1 ms, 0.1-5 V at a rate of 60 bpm)	Electro-physiological properties	–

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
			Rotating-wall bioreactor (dynamic laminar flow, 12 rpm) Electrophysiological assessment apparatus			
Carrier *et al.*, 2002 [63, 94]	Neonatal rat cardiac myocytes	Porous, disk-shaped, nonwoven mesh of fibrous PGA	Flasks (stirred turbulent flow, 50 rpm) Direct perfused cartridges (1h at 0.2 ml/min, and then 10 days at 0.6, 1 or 3 ml/min) Electrophysiological assessment apparatus	–	Culture parameters (pH, pO_2, pCO_2)	–

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Radisic *et al*., 2003 [95]	Neonatal rat cardiac myocytes (feasibility studies) Murine C2C12 cells (detailed seeding studies)	Collagen sponges enriched with Matrigel	Orbitally mixed dishes (25 rpm) Direct perfused cartridges (seeding: alternating flow; cultivation in a perfusion loop: unidirectional flow, 0.5 ml/min) Electrophysiological assessment system	Electrical square pulses (2 ms, 1-5 V, 1Hz)	Contractile activity	–
Radisic *et al*., 2004 [96]	Neonatal rat cardiac myocytes	Collagen sponges enriched with Matrigel	Electrical stimulation bioreactor (orbitally mixed flow, 25 rpm)	Electrical rectangular pulse (2 ms, 5 V/cm, 1Hz)	Contractile activity (real time) Electrical activity	–
Tandon *et al*., 2008 [85], 2009 [60], 2011[97]	Neonatal rat cardiac myocytes	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*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Tandon *et al*., 2009 [98]	Human adipose tissue-derived SCs 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 [99]	Neonatal rat cardiac myocytes	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 [100]	Neonatal rat cardiac cells	Channeled PGS	Perfused	Electrical square monophasic pulses (3 V/cm, 3 Hz)	Contractile activity	-
Hansen *et al*., 2010 [101]	Neonatal rat cardiac myocytes	Cell/fibrinogen/ Matrigel/ thrombin mixture	Miniaturized 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

(***Continued***)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
Schaaf *et al.*, 2012 [47]	Human embryonic SC-derived cardiac myocytes	Cell/fibrinogen/ Matrigel/thrombin mixture	Miniaturized drug screening platform (static flow)	-	Contractile activity (real time)	Force: 0.12 mN/mm^2 Frequency: 0.5 Hz
Kensah *et al.*, 2011 [102]	Neonatal rat cardiac myocytes	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: 0.96±0.09 mN (controls) 1.42 ± 0.09 mN (stretch alone) 2.54 ± 0.11 mN (noradrenalin)
Kensah *et al.*, 2012 [103]	Murine pluripotent SC-derived cardiac myocytes Human embryonic and pluripotent SC-derived cardiac myocytes	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: 0.97 ± 0.08 mN (static stretch) 0.77 ± 0.07 mN (cyclic stretch) 1.22-1.42 mN (ascorbic acid + growing static stretch)

(*Continued*)

Table 2. Devices and bioreactors for cardiac tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Physical stimulation	Monitoring	Contraction forces
						Maximum specific active force: 4.4 mN/mm^2 (human)
Boudou *et al*., 2012 [104]	Neonatal rat cardiac myocytes	Collagen/fibrin 3D micropatterned matrices	MEMS cantilevers	Electrical biphasic square pulses (1ms, 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.

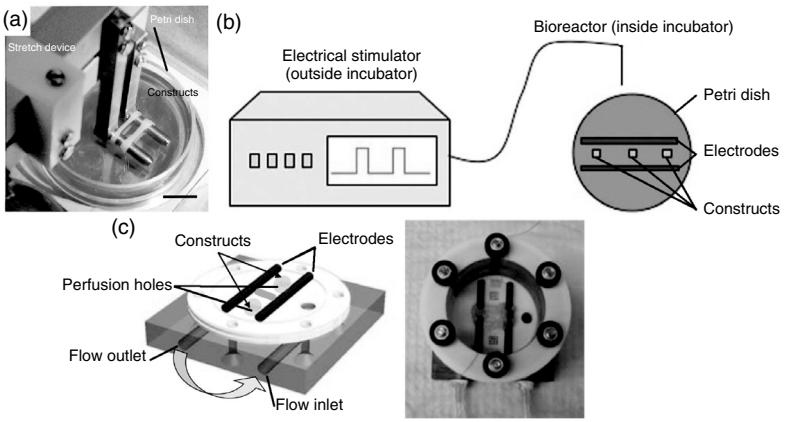

Figure 2. (a) Stretch device developed by Eschenhagen [105]. (b) Bioreactor for electrical stimulation developed by Tandon [60]. (c) Perfusion bioreactor developed by Maidhof [100]. Figures reproduced with permissions.

Eschenhagen, Zimmermann and coworkers [5, 105, 106] proved the *in vivo* feasibility of the EHT implantation on rats. Implanted EHTs maintained a network of differentiated cardiac myocytes and were strongly vascularized [5, 105, 106]. Moreover, EHTs grafted on the heart of syngenic rats [106] preserved contractile function *in vivo*. More recently, Zimmermann *et al*. [69] evaluated the performance of multiloop EHTs implanted in Wistar rat heart muscles after myocardial infarction. 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*. [87] developed a bioartificial engineered cardiac construct (ECC) capable of synchronized multidirectional contraction. Based on previous studies [68, 91], ECCs were prepared mixing neonatal rat cardiomyocytes 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*. [92] 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 cardiomyocytes 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 cardiac myocytes 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 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 cardiomyocytes on cell-supports within rotating bioreactor (HARV, Synthecon), Akins *et al*. [93] 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*. [35] 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 ultra-structural features peculiar of native cardiac tissue.

Papadaki *et al*. [89] cultivated highly concentrated neonatal rat cardiac myocytes, seeded on laminin-coated PGA scaffolds, within rotating bioreactors. By using a specific apparatus [107], 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 cardiac myocytes 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 [63] and oxygen concentration [94] on engineered cardiac tissues obtained from fibrous PGA scaffolds seeded with neonatal rat cardiac myocytes. 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 [63]. It was also proven that a marked positive correlation exists between medium pO_2 and the aerobicity of cell metabolism, DNA and protein content, and the expression of cardiac-specific markers [94]. However, the system in [94] 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 [35, 63, 94]. To improve cell density Radisic *et al*. [95, 108] developed a new seeding strategy within the same perfusion system as in [94]. Using Matrigel as vehicle for cell delivery, neonatal rat cardiomyocytes 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 cardiomyocytes 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 [109, 110] 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 [96] subjected cardiac constructs cultured *in vitro* (prepared as in [95]) 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 [96]. Adopting a similar apparatus (Figure 2B), further studies on electrical stimulation were performed by Tandon and coworkers [85]. Cardiac constructs were prepared as previously described [96, 108] 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 cardiomyocytes were observed. In more recent studies [60, 97], 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*. [98] 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 fibroblast growth factor.

Recently, Barash *et al*. [99] 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 [99] was taken by Maidhof *et al*. [100], who designed a bioreactor (Figure 2C) 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*. [101] 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*. [47] generated fibrin-based human EHTs (hEHTs) from an unselected population of differentiated human embryonic SCs containing 30-40% a-actinin-positive cardiac myocytes. Constructs displayed a dense network of longitudinally oriented, interconnected and cross-striated cardiomyocytes 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 [102] 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 cardiomyocytes mixed with type I collagen and Matrigel, according to [91]. 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 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*. [103] cultured highly purified murine and human pluripotent SC-derived cardiomyocytes to generate functional BCTs and to investigate the role of fibroblasts, 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 lead 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/mm^2 in human BCTs, only 3- to 5-fold lower than active forces reported for native human myocardium [111, 112].

In the field of scaffold design for *in situ* cardiac repair, the bioreactor proposed by Kensah [102, 103] allowed Dahlmann *et al*. [113] 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 cardiomyocyte-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*. [104] employed microelectromechanical 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*. [104] 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.

4.2. Bioreactors for Vascular Tissue Engineering

The major challenge for clinical application of vascular TE is the development of small ID vascular grafts for the coronary and peripheral vasculature [43, 114, 115, 116, 117, 118, 119], characterized by the presence of a confluent endothelium and differentiated, quiescent SMCs, and providing evidence of mechanical and biological properties for adequate suture retention at implantation and endurance at systemic arterial pressures [116, 120]. In detail, the range of vessel diameters suitable to be engineered *in vitro* is 1-6 mm for veins and 1-8 mm for arteries [121]. As for the treatment of vessels with diameter smaller than 1 mm, microvascular surgery techniques [122] as well as angiogenic approaches for promoting the formation of new capillary networks from existent microvasculature [123] are widely adopted.

In the past decade, a huge amount of bioreactor-based applications were developed, which have brought vascular TE closer to the clinical application. The following studies, described in detail and ordered with respect to flow conditioning and perfusion in Table 3, demonstrate that adequate combinations of vascular cells, scaffolding, and signaling within dedicated bioreactors have led to the generation of biologically active vessels, thus offering the potential for permanent and effective treatments of many vascular diseases.

By adopting a fixed-wall bioreactor, in 1986, Weinberg and Bell [124] produced the first engineered vascular graft model from a mixed population of bovine aortic ECs, SMCs, and adventitial fibroblasts seeded on collagen, reinforced with open Dacron mesh sleeves. The graft presented a multilayered structure, similar to a mammalian muscular artery; however SMCs and collagen fibers were oriented longitudinally rather

Table 3. Bioreactors for vascular tissue engineering

Authors	Cells	Scaffold	Device	Flow Conditioning	Perfusion	Monitoring	Vessel size
Weinberg and Bell, 1986 [124]	Bovine aortic ECs, SMCs and adventitial fibroblasts	Collagen and Dacron mesh	Fixed-wall bioreactor	-	-	Burst strength	-
L'Heureux *et al*., 1998 [125]	Human umbilical vein SMCs, HUVECs, and human skin fibroblasts	-	Fixed-wall bioreactor	-	Luminal flow	Burst strength	ID: 3 mm Length: 5 cm
Nasseri *et al*., 2003 [84]	Ovine vascular myofibroblasts	PGA-based, coated with collagen	Rotating-wall hybridization oven	Continuous flow	Luminal flow	-	Large tubes ID: 12 mm Length: 6 cm Small tubes ID: 5 mm Length: 2 cm
Mironov *et al*.,2003 [59]	-	Bovine carotid arteries Silicon tubes	Fixed-wall bioreactor	Continuous or pulsatile flow	Luminal and extraluminal flow	Intraluminal pressure and radius (real time) Burst strength, strain	ID: 2-6 mm
Geeslin *et al*., 2011 [126]	Rat ECs and SMCs	Decellularized rat aorta matrix	Rotating-wall bioreactor	Continuous flow	Luminal and extraluminal flow	-	-

(***Continued***)

Table 3. Bioreactors for vascular tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Flow Conditioning	Perfusion	Monitoring	Vessel size
Niklason *et al*, 1999 [120]	Bovine aortic SMCs and ECs	PGA	Fixed-wall bioreactor	Pulsatile flow	Luminal flow	Intraluminal pressure Stress-strain relationship	-
Hoerstrup *et al*., 2001 [114]	Ovine carotid artery myofibroblasts and ECs	PGA coated with P4HB	Fixed-wall bioreactor	Pulsatile flow	Luminal flow	Burst strength	ID: 5 mm
Williams and Wick, 2004 [127]	Bovine aortic SMCs and ECs	Porous tubular PLA nonwoven	Fixed-wall bioreactor	Pulsatile flow	Luminal flow	Inlet and outlet flow rate (real time)	ID: 4.5 mm Length: 5 cm
Narita *et al*., 2004 [128]	Canine aortic SMCs, ECs, and myofibroblasts	Porous tubular PLA nonwoven	Fixed-wall bioreactor	Pulsatile flow	Luminal flow	Pressure and flow rate (real time)	ID: 2 cm Length: 8 cm Wall thickness: 1 mm
Yazdani *et al*., 2009 [129]	Rat aortic vascular SMCs	Decellularized porcine carotid arteries	Fixed-wall bioreactor	Pulsatile flow	Luminal flow	Flow rate (real time)	ID: 3-4 mm Length: 5 cm
Bilodeau *et al*., 2005 [130]	-	-	Rotating-wall	Pulsatile flow	Luminal and extraluminal flow	Construct diameter (real time) Burst strength	Average radius: 3 mm Length: 5 cm Wall thickness: 1mm

(***Continued***)

Table 3. Bioreactors for vascular tissue engineering (*Continued*)

Authors	Cells	Scaffold	Device	Flow Conditioning	Perfusion	Monitoring	Vessel size
Zhang *et al*., 2009 [131]	Human coronary artery SMCs and human aortic ECs	Tubular electrospun silk fibroin tubes	Rotating-wall bioreactor	Pulsatile flow	Luminal and extraluminal flow	-	ID: 3 mm Length: 5 cm Wall thickness: 120 μm
Amensag *et al*., 2012 [123]	HUVECs and human vascular SMCs	Decellularized human amniotic membrane	Fixed-wall bioreactor	Pulsatile flow	Luminal and extraluminal flow	-	-
Couet *et al*., 2012 [133]	-	-	Fixed-wall bioreactor	Pulsatile flow	Luminal and extraluminal flow	Pressure Flow rate Construct diameter Elastic Elastic modulus Shear stress Strain (real time)	-

HUVECs: human umbilical vein endothelial cells; P4HB: poly-4-hydroxybutyrate; PLA: poly-lactid acid.

than circumferentially, with poor and unstable mechanical properties (maximum burst strength around 323 mmHg).

More encouraging results were achieved by L'Heureux *et al*. [125], who developed a cylindrical three-layered construct by sequentially wrapping different cellular sheets, seeded with human umbilical vein SMCs, human umbilical vein endothelial cells (HUVECs) and human skin fibroblasts, and cultured in a fixed-wall bioreactor. The construct was characterized by histological organization of native tissue, high burst strength (2000 mmHg), positive surgical handling, and a functional endothelium. These results were confirmed by *in vivo* tests performed to assess suturability and early mechanical stability of the construct.

Using continuous flow and perfusion of the vessel lumen, in 2003, Nasseri *et al*. [84] developed a device for rotational seeding and culturing of ovine vascular myofibroblasts seeded onto biodegradable polymer scaffolds, suitable for small- (ID: 5 mm, length: 2 cm) and large- (ID: 12 mm, length: 6 cm) diameter blood vessels. Continuous flow and mass transfer enhanced by rotation promoted cell adhesion, tissue growth, and the formation of confluent layers of myofibroblasts on both inner and outer surfaces of the constructs.

Based on the knowledge that arterial growth during embryonic development is associated with longitudinal strain, Mironov *et al*. [59] developed a bioreactor combining perfusion, with continuous or pulsatile flow, and functional capacity for longitudinal strain. Periodic variations of longitudinal strain (0-200%) were applied to silicone tubes and natural bovine carotid arteries (ID: 2–6 mm), adopted as models of vascular grafts. A pressure transducer and a digital camera monitored the biomechanical properties of the construct, showing that pressure-circumferential strain of the construct had a non-linear relationship, and the diameter decreased with the longitudinal strain.

Imposing similar perfusion conditions, Geeslin *et al*. [126] designed a bioreactor for the reconstitution of a decellularized vascular matrix. Within the bioreactor, the graft was supported at its ends and rotated to guarantee a uniform coating of both the interior surface (with ECs) and the exterior surface (with SMCs) of the decellularized matrix. Preliminary tests, performed with a decellularized rat aorta recellularized with rat ECs at the luminal surface and rat SMCs injected in the culture chamber, showed a uniform cell coverage, with recellularization achieved both on interior and exterior surfaces of the reconstituted matrix, demonstrating the potency of the proposed dynamic culture conditions.

Niklason *et al*. [120] seeded autologous bovine aortic vascular SMCs and ECs on highly porous PGA scaffolds and subjected the constructs to physiologically pulsatile flow (165 bpm). After cultivation, the gross appearance of the vessel was identical to that of bovine native arteries, and pulsatile stress significantly increased suture retention strength, wall thickness, and collagen content to values comparable to that of native vessel (burst pressure > 2000 mmHg). Implanted into the right saphenous artery of miniature swine, the constructs demonstrated good flow at implantation and remained open at least for 2 weeks postoperatively.

In 2001, Hoerstrup and coworkers [114] developed an *in vitro* pulse perfusion duplicator system. Dynamically cultured constructs, prepared by PGA coated with

P4HB (poly-4-hydroxybutyrate) and seeded with ovine myofibroblasts and ECs (ID: 5 mm), exhibited confluent smooth inner surfaces, a higher value of burst strength (326 mmHg) and a suture retention strength five times greater than statically cultured constructs, with tissue organization and mechanical properties appropriate for surgical implantation.

Williams and Wick [127] proposed a modular bioreactor for sequential seeding of bovine SMCs and ECs onto porous tubular PLA (poly-lactid acid) nonwoven scaffolds (ID: 4.5 mm, length: 5 cm), providing pulsatile flow and monitoring tissue growth and maturation. Dynamic culture enhanced cell proliferation, expression of differentiated phenotype by SMCs, and ECM deposition. Seeding of ECs after SMCs generated a confluent monolayer in the lumen.

Similar results were reported by Narita *et al*. [128], who developed a bioreactor providing a wide range of pulsatile flows with a completely physiological pressure profile. Biodegradable nonwoven PLA tubes (ID: 2 cm, length: 80 mm, thickness: 1 mm) were seeded with canine SMCs, ECs and myofibroblasts and cultured under physiological pulsatile pressure. The constructs presented marked total protein content and high cell number, with cells widely distributed and in close contact with each other.

In 2009, Yazdani *et al*. [129] proposed a system for seeding, proliferation, and maturation of rat vascular SMCs seeded on decellularized porcine carotid arteries (ID: 3-4 mm, length: 5 cm). Recellularized engineered vessels were subjected to pulsatile flow regimes (1 Hz). Cyclic bioreactor conditioning resulted in increased SMC proliferation and accelerated the formation of a significant muscular layer.

Bilodeau *et al*. [130] combined pulsatile flow with perfusion inside and outside the lumen. They designed a bioreactor (Figure 3a) for 3D regeneration of arterial tissue (average radius: 3 mm, length: 5 cm, wall thickness: 1 mm) on a cylindrical scaffold. Once seeded, the inner and outer side of the scaffold were perfused by culture medium thanks to the rotation of the construct along an horizontal axis. During cell culture, parameters such as internal flow and stretching of the vessel could be tuned, mimicking the gradual maturation evolution.

Zhang *et al*. [131] developed an engineered vascular construct using tubular electrospun silk fibroin scaffolds (ID: 3 mm, length: 5 cm, wall thickness: 120 μm) sequentially seeded with human SMCs and human ECs, and cultivated under physiological pulsatile flow within a dedicated bioreactor (Figure 3B). Dynamic culture conditions improved mass transport, and enhanced tissue formation, metabolic activity, cell alignment, and the retention of differentiated cell phenotype.

In 2012, Amensag *et al*. [132] proposed a multilaminate rolling approach, using a decellularized human amnion, for the generation of tubular cell-dense constructs which can be manufactured into different sizes to suit specific applications. Dual perfusion vascular bioreactors, to isolate lumen and ablumenal circuits, were employed for seeding HUVECs and human vascular SMCs. Cell-seeded sheets were rolled around a mandrel to form a tubular construct with concentric layers of cells between each amnion layer. Graft mechanical properties were controlled by modulating the number of layers, allowing to match tensile properties and compliance values. Histological assessments showed tightly bound structures forming uniform tubular constructs.

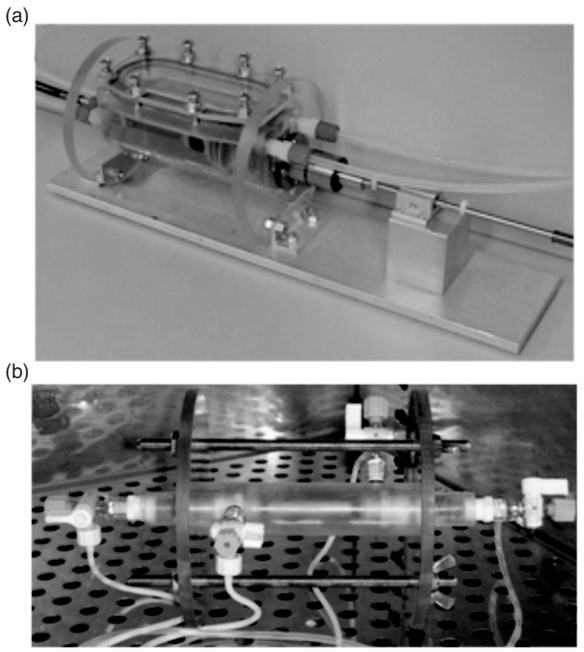

Figure 3. Dual closed-loop perfusion bioreactors by (a) Bilodeau et al. [130], and (b) Zhang et al. [131]. Reproduced with permissions.

Finally, a new approach inspired by fetal development, mechanobiology and optimal control paradigms was proposed by Couet *et al*. [133]. A bioreactor system for measurement and control in real time of culture parameters and continuous modeling and optimization of TE processes was designed. Culture conditions can be adapted to the maturation state of the tissues, in order to maximize the efficiency of the regeneration process by shortening the production of tissues, and to acquire knowledge about the growth and remodeling process.

5. DISCUSSION AND FUTURE DIRECTIONS

Based on the reviewed literature, it is evident that there are increasing efforts in developing *in vitro* strategies and methods for assessing fundamental myocardial and vascular biology and physiology. In addition, recently, there is an increasing interest in generation of engineered cardiac and vascular 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 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 cardiovascular substitutes.

Recently, bioreactor-based platforms were also designed for drug screening based on the effects of pharmacological compounds on biological tissues, and, in combination with the recent induced pluripotent SC technology, for disease modeling as physiological models of human disease processes that might be prevented or treated if better understood. The *in vitro* generation of functional engineered cardiovascular tissues with morphological and physiological properties of native heart and vessels suitable for clinical applications still represents a challenge, but the massive research in recent years provided a significant advance in this direction.

In cardiac TE, recent studies demonstrated that the key modulators of engineered myocardium are not only the cell composition, differentiation, and orientation, but also the composition of the embedding matrix and external stimuli such as cyclic stretching or electrical stimulation. Therefore, bioreactors delivering native-like cyclic mechanical stretch and electrophysiological stimulation, applied individually or in combination, markedly contribute to the development of functional engineered constructs.

By imposing physiological stimulations, it is possible to improve cell differentiation and structural and functional properties of the construct [5, 80, 87, 90, 91, 92, 102, 105, 106]. 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 constructs [80, 87, 92, 106]. 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 [69]. 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 [103].

Perfusion, by reducing diffusional gradients associated with mass transport over macroscopic distances and improving control of local levels of pH and oxygen, improves cell seeding efficiency throughout the thickness of 3D scaffolds, promotes homogeneous distribution of oxygen within the culture chamber, increases construct thickness, enhances the expression of cardiac-specific markers [35, 63, 94, 95], and guarantees high cell viability by protecting cells from critical hydrodynamic shear [134].

Through physiological electrical field stimuli, ultra-structural differentiation and morphological and constitutive hallmarks of maturing cardiomyocytes can be obtained. In particular, 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 SCs, and promote disassembly of gap junctions [98], whereas native-like electrical pulses improve contractility with a marked level of ultra-structural differentiation [85, 96, 97, 98]. Both DC and electrical pulse stimulations upregulate the expression of connexin-43 [85, 96, 97, 98]. The combination of mechanical and electrical stimulations promotes the

electrical interconnection through gap junctions of cardiomyocytes, promoting the production of an engineered tissue that presents conduction velocity and spatial distribution of connexin-43 as to the native one [89, 100]. Finally, the combined use of perfusion and electrical stimulation can induce cell alignment and coupling, increase the amplitude of synchronous construct contractions and the number of living cells, and enhance the level of ultra-structural organization and cell viability [96, 99, 108, 110].

No robust methods currently exist to measure both fine-scale cytoskeletal and extracellular architecture as well as cardiac contractility, the ultimate functional output of the myocardium. Moreover, due to their size, centimeter-scale constructs are too expensive to be generated in a high-throughput manner and require histological sectioning to visualize cellular and extracellular architecture. Therefore recently, for testing biophysical and chemical culture parameters in a high-throughput and combinatorial manner, miniaturized screening platforms were developed [101, 102, 103, 104]. These systems are suitable for drug screening [47] as well as for optimization of culture conditions, ECM design [104, 113], and compensate current challenges in up-scaling of pluripotent SC-derived cardiomyocytes [103] in the field of disease modeling.

Concerning vascular TE, the complex layered structure of blood vessels imposes the use of bioreactor solutions for guaranteeing adequate oxygen and shear stress distributions for the production of effective substitutes. Perfusion (inside and/or outside of the vessel lumen), physiological pulsatile flow, rotating culture chamber and provision of adequate mechanical stress, used individually or in combination, are the solutions adopted for achieving this goal. In particular, perfusion, combined with rotational seeding and culturing, enables homogeneous oxygen and shear stress distributions around the inner and outer area of the construct, promoting the attachment and proliferation of cells on luminal and exterior surfaces [84, 126]. Continuous perfusion in combination with variations of longitudinal strain promotes vascular wall maturation as it happens during vascular development at the embryonic stage [59]. By imposing physiological pulsatile flows and adequate mechanical stress, morphological and physical properties of native arteries can be achieved with enhanced cell proliferation and protein content [114, 120, 127, 128, 129, 132]. Finally, the combination of pulsatile flow, perfusion inside and outside the lumen, and rotating vessel systems improves mass transport and aerobic cell metabolism, and enhances tissue formation, ECM production, cell alignment, and the retention of differentiated cell phenotype [130, 131].

This review study clearly indicates that specific physical stimuli are essential for cell differentiation and maturation of cardiovascular substitutes with morphological and physical properties of native tissues, and bioreactors are the technological solution to provide these stimuli in a controlled and automated manner.

In fact, in the process of engineering biological constructs, bioreactors allow to (1) promote uniform and effective cell seeding of 3D constructs, by providing dynamic perfusion within a 3D environment, (2) enhance nutrient and gas transport and distribution, with a suitable waste removal, making up for the role of native vascular network, (3) provide physiological physical stimuli that simulate the native

environment, and (4) tightly monitor and control the culture parameters in order to maximize the efficiency of the TE process. However bioreactor technology is still in evolution, with several limitations to be addressed. Nowadays, even by using a bioreactor, vascularization and innervation have not yet been achieved in cardiovascular engineered constructs, although these are fundamental issues for a functional integration of the graft with host blood supply and tissues. Furthermore, the supply of oxygen and soluble nutrients is still limited for *in vitro* culture of 3D thick tissues. These disadvantages represent the greatest challenges to be addressed in order to definitively move cardiovascular TE from bench to clinical practice.

In order to overcome these limitations, a more in-depth understanding of the exact physiological conditions to be reproduced, together with a series of technological improvements not yet attained, could substantially help. In this effort, significant advantages 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 O_2 and CO_2, metabolite/catabolite concentrations, sterility, etc.), monitoring function and structure (e.g.: stiffness, force, strength, permeability, composition of the scaffold, cell number, cell viability, etc.) of developing engineered cardiovascular constructs still remains a relatively unexplored area and a highly challenging field of research [81]. For example, although contractile function is one of the most important outputs of engineered heart 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 [68, 69, 80, 90, 95, 105, 106]. 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 [102]) or indirect (using optical analysis [101, 104]) measurement of the contraction forces, providing noninvasive on-line monitoring during prolonged culture.

Real-time monitoring could allow to investigate the cellular response to specific culture conditions and to identify the still unknown mechanisms of the cardiovascular tissue regeneration, while the possibility to adapt the culture parameters to the maturation phase of the construct could enhance the efficiency of the regeneration process [133]. Moreover, the possibility to obtain quantitative data without interrupting the experiment reduces operator-dependent errors and contamination risk, ultimately enhancing automation and repeatability of the manufacturing process in terms of traceability and safety of the process itself, allowing functional quality control of engineered tissues. In this scenario, an optical access could be useful for directly observing the construct behavior [135, 136, 137] and performing functional imaging [15] with a non-invasive on-line monitoring during prolonged culture. Advanced technological techniques, such as Coherent anti-Stokes Raman scattering (CARS) microscopy in conjunction with second harmonic generation (SHG), could be used to perform noninvasive analysis and to obtain simultaneous imaging of cell morphology

and distribution of ECM components throughout the 3D construct [138]. Moreover, bioreactors should be equipped with specific sensors and control system networked together for the on-line, high throughput monitoring of basic parameters such as temperature, pO_2 and pCO_2, in addition to quantities that provide quantitative information on gene expression, cell metabolism, contraction force and impulse propagation [133], going beyond subjective and qualitative conclusions [82]. This scenario could also include the so-called software sensors, recently introduced by Couet *et al*. [133], that adopt mathematical or numerical models to provide an evaluation of a missing measurement, such as elastic modulus or shear stress, that relates the desired information with other measurements that can be taken online. Further improvements can come from analytical tools such as computational fluid dynamic [139, 140, 141] and mathematical models [142], used to assess *a priori* the optimal design and culture conditions for the bioreactor. Finally, very recently, micro-bioreactors have been developed to overcome the conventional bioreactors disadvantage of large operating volumes, which is a serious limitation in studies involving the use of expensive media components [101, 102, 103, 104, 143].

Besides technical considerations, regulatory and manufacturing issues also represent challenges to successfully translate cardiovascular TE technologies from bench to bedside. The clinical efficacy of a tissue-engineered product will need to be accompanied by a (1) cost-effective manufacturing process and cost-benefits over existing therapies, (2) absolute safety for patients, manufacturers, and the environment, and (3) compliance to the evolving regulatory framework in terms of quality control and good manufacturing practice requirements. For these reasons, a closed, standardized, and operator-independent system would assure great benefits in terms of safety and regulatory compliance, thus improving the cost-effectiveness of a manufacturing process, and maximizing the potential for large-scale production in the long-term [81].

6. CONCLUSIONS

In cardiovascular TE, bioreactors are fundamental tools for (1) investigating the maturation of cardiovascular engineered tissues, since they provide a comprehensive level of monitoring and control over specific environmental factors in 3D cultures, and (2) promoting the *in vitro* generation of functional cardiovascular substitutes. The high level of automation that can be achieved by bioreactors will allow not only to perform more controlled, reproducible and statistically significant tests, but in a prospective vision, it will be essential in routine productions for clinical applications. In fact, automation allows to improve quality, process safety, and production volume, lowering risk factors and production costs.

In the near future, with exciting and promising research advancements, it is expected that the increasing interaction among scientists, engineers, and clinicians will translate the research-based field of cardiovascular TE into clinical practice. In this process, the transition from bench to bedside will require a switch from highly flexible bioreactors to specialized bioreactors, implementing the defined bioprocesses in a standardized way. The resulting devices will become a key factor and synonymous of advanced systems for the development of automated, monitored, standardized, traceable, cost-

effective, and safe manufacturing processes of production of structurally and functionally well-defined 3D cardiovascular engineered tissues for large-scale clinical applications, overcoming limitations of conventional manual techniques and bridging the gap between healthcare and engineering.

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CONFLICT OF INTEREST

The authors indicated no potential conflicts of interest.

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