Hydrodynamics and bioprocess considerations in designing bioreactors for cardiac tissue engineering

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Abstract

One of the most attractive fields in regenerative medicine and tissue engineering is cardiac cell regeneration due to the limited capacity of the heart muscle to regenerate itself. Recent advances in the field of stem cell bioprocess technology and biomaterials have accelerated progress in creating three-dimensional (3D) cardiac engineered tissues in vitro. Control of bulk and microscopic transport efficiency, which is determined by the choice of the bioreactor design and substrate material, poses direct influence on cell viability, proliferation and differentiation in vitro. This paper provides a critical review on the hydrodynamic considerations with respect to design aspects of the bioreactor and selection of substrate material for 3D cardiac tissue engineering.

Keywords: shear stress, flow dynamics, porosity, topography, stiffness, proliferation, differentiation

Introduction

Cardiovascular disease (CVD) is a leading cause of mortality in most developed countries accounting for 30% of all deaths worldwide in 2008 [1]. Current trends in obesity and the lack of a healthy lifestyle will likely lead to an increase in total population suffering from heart diseases, causing associated healthcare costs to increase in the United States [2]. Coronary heart disease accounts for 42% of the total mortality rates due to CVD. The narrowing of blood vessels in coronary heart disease patients limits the supply of blood to the heart and leads to myocardial ischemia. During myocardial ischemia, lack of oxygen and nutrient supply to cardiac muscle cells results in cell death of functional cardiomyocytes. The infarcted area undergoes inflammation and forms collagenous scar tissue, which lacks cardiac muscle cell functionality, thus the infarcted area will not be able to perform functions such as muscle contraction and electrical conduction. This will lead to decreased working capacity of the ventricular heart and increases the risk of heart failure. Current clinical interventions are still unable to restore the full function in the infarcted area and for patients who suffer from severe heart damage, the current option is to receive heart transplantation but the availability of heart donors is very scarce.

Cardiac tissue engineering offers a promising cell-based therapy to repair damaged heart muscles and achieve cardiac regeneration. However, current challenges remain in the generation of clinically relevant cell numbers, cardiac functionality and tissue integration. In therapeutic applications, local administration of 10^8–10^9 total cell numbers of functional cardiomyocytes is often necessary to replace damaged ventricular cardiomyocytes in a post-infarct patient [3]. Conventional in vitro two-dimensional (2D) methods using tissue culture polystyrene (TCP) can be impractical for large-scale production. Moreover, cells cultured on TCP suffer from altered morphologies when they adhere to the hard surfaces. This can lead to unnatural cell behaviors that can differ significantly from the in vivo environment, which involves three-dimensional (3D) cell-cell and cell-matrix interactions. To this end, various groups have looked at developing 3D culture systems in the effort to replicate the physical and structural components of the in vivo microenvironment. In cardiac tissue engineering, the goal is therefore to design 3D environments that would bear greater resemblance to cardiomyocytes in the myocardium than 2D cultures [4-5].

In order to readily scale-up and achieve clinically relevant cell numbers, the use of bioreactors is necessary. In the culture of adherent cell types such as cardiomyocytes, cultures in bioreactors often requires the use of 3D scaffolds to achieve cell numbers, cell densities and morphological mimicry to those in the in vivo state. Several bioreactor cultures have successfully produced large numbers of cardiomyocytes in the magnitude of 10^8 cell numbers [6-7]. However, hydrodynamics and scaffold properties should be considered in the design of bioreactors. Many different approaches were taken to address these issues independently. In this review, we discuss in greater detail the hydrodynamic and bioprocess considerations involved in the

design of a scalable bioreactor and substrate architecture relevant to cardiac tissue engineering.

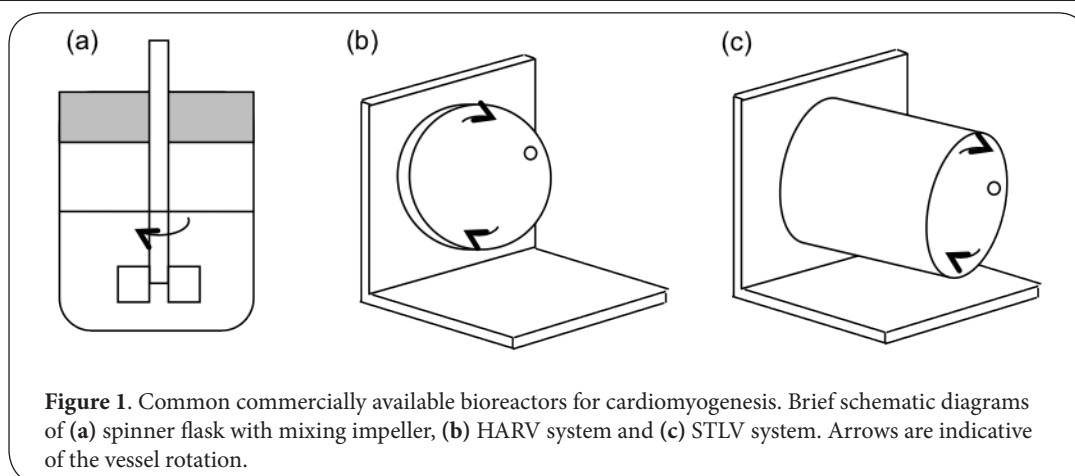## Sources for regenerative therapies of cardiomyocytes

One of the first considerations for cardiac tissue engineering is the choice of cell source. The major types include mature cells, neonatal cells, adult stem cells and embryonic/induced pluripotent stem cells. Mature cardiomyocytes are the ideal cell source for their functional utility but the sourcing of these cells can be rather challenging. Mature cardiomyocytes also have very limited proliferative capacity, and this limits the cell quantity we can obtain to replace the infarcted area efficiently [8]. In addition, mature cardiomyocytes do not have the ability to repair damaged cardiomyocytes in the infarcted area. Thus, cell types with greater proliferative capacity, such as stem cells and progenitor cells, present greater promise as good cell sources of cardiac cell regeneration. Adult skeletal myoblasts, which are precursors of myocytes, have better proliferative capacities than mature cardiomyocytes and still contain contractile apparatus and lineage specificity [9]. Myoblasts can be isolated from the patient's own tissue, enabling autologous transplantation. However, as they derive from the skeletal muscle lineage, these cells still show that they are unable to integrate with the myocardium and result in problems like arrhythmias and fibrosis [10-11].

Adult stem cells are another potential source for cardiomyocytes. Cardiac stem cells (CSCs) and cardiac progenitor cells (CPCs) have the advantage of being primed to be cardiac-specific and are suitable candidates for regenerating the myocardium. They can form aggregates called cardiospheres and give rise to cardiomyocytes, smooth muscle cells and endothelial cells [12]. These cell types have demonstrated good potential for myocardial regeneration *in vivo* with improved cardiac functionality observed upon transplantation of the cardiospheres [13]. Cardiospheres and their derived cells are an attractive source for cardiac regeneration due to their high cardiogenic lineage specificity controllable under both *in vitro* and *in vivo* conditions while maintaining high self-renewal ability as immature progenitors [14-16]. However, it is hypothesized that their cardiac regenerative ability is not due to direct cardiomyogenesis of the transplanted cardiospheres but instead due to an indirect mechanism inducing endogenous regeneration of the host's myocardium [17]. Another study suggested that cardiomyogenesis observed by the cardiospheres could be due to myocardial tissue fragments, which were not thoroughly removed during the isolation process [18]. Thus, more studies are still required to determine their candidacy at this time. Despite their suitability as good cell source, isolation and expansion of these cells are complex processes. Challenges faced in cell expansion pose limitations to sufficient production of cardiomyocytes. Cardiac-derived adherent proliferating (CAP) cells, obtained from endomyocardial biopsies are easier to isolate and expand due to their higher proliferating capacity

[19]. These cells are also primed for cardiac specificity but so far initial studies indicate that they exhibit a preference for angiogenesis over cardiomyogenesis.

Another common source of adult stem cells is the bone marrow and umbilical cord; they comprise of two main types: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). A few studies have shown that HSCs from bone marrow and umbilical cord blood may have cardiac regenerative potential [20-22]. In an *in vivo* study performed in mice, Jackson *et al.,* showed that the highly enriched side population (SP) cells engrafted and differentiated into cardiomyocytes and endothelial cells *in vivo* [20]. In an *in vitro* study, Xaymardan *et al.,* identified the bone marrow derived c-kit$^+$ cells as the cell population with an ability to form beating clusters in the presence of vascular endothelial and fibroblast growth factors [21]. Yet, other studies have contradicted these observations and showed that HSCs do not readily transdifferentiate into cardiomyocytes [23-24] and that their beneficial effects in transplantation studies were mostly due to cell fusion [25-26], thus they may have limited cardiac regenerative capacity. Given the contradicting observations made, therapeutic applications of HSCs for cardiac regeneration remain uncertain. Even if these cells were able to transdifferentaite into cardiomyocytes, they are very limited in cell numbers and may not be sufficient to make significant improvements in cardiac functionality.

MSCs remain the predominant source of adult stem cells supporting hematopoiesis and the maintenance of mesenchymal tissues [27]. MSCs from both bone marrow (BM-MSCs) and umbilical cord have demonstrated an ability to differentiate into osteogenic, adiopogenic, chondrogenic and cardiomyogenic lineages [28-29]. Human BM-MSCs can be used as allogeneic or autologous cell therapy [30-32]. One distinct advantage of BM-MSCs is their ability to inhibit inflammation and evade immune detection, which can then prevent rejection and improve recovery [33-36]. Despite these benefits, generation of cardiomyocytes from both human and non-human MSCs have not produced significant yields [37-39]. Endogenous myocardial regeneration using MSCs have not clearly demonstrated an ability to generate contractile tissue *in vivo* but have demonstrated safety and clinical benefits of the cell therapy [40-42]. However, more studies in quantifying the potential clinical benefit and elucidating the mechanism of action are necessary. The extraction of MSCs from bone marrow is also highly invasive and painful. On the other hand, MSCs derived from umbilical cord are extracted from the placenta or cord lining. Preclinical studies in placental derived MSCs showed its potential therapeutic utility for dilated cardiomyopathy [43] and its ability to differentiate into various cell types including cardiomyocytes [44]. Umbilical cord derived MSCs demonstrate potential clinical utility but are equally challenged in cell numbers. Another group of adult stem cells, which could be extracted via relatively non-invasive methods from abundant tissue, is adipose-derived stem cells (ASCs). So far, *in vitro* and *in vivo* evidences indicate the

Figure 1. Common commercially available bioreactors for cardiomyogenesis. Brief schematic diagrams of **(a)** spinner flask with mixing impeller, **(b)** HARV system and **(c)** STLV system. Arrows are indicative of the vessel rotation.

ability of ASCs to differentiate into cardiomyocyte-like tissue [**45-47**]. ASCs demonstrate a potential utility for cardiomyocyte regeneration but more extensive studies are necessary to determine its candidacy at this time.

Embryonic stem cells (ESCs) represent a highly proliferative and pluripotent cell source that is able to generate high cell numbers. Among the various cell lineages, cardiomyocytes were reported as one of the first few cell populations identified that spontaneously differentiated *in vitro* [**48**]. In addition, ESCs are the only cell source that demonstrated equal tendencies in differentiation towards cardiogenesis and vasculogenesis. Such a characteristic can be highly beneficial to the regeneration of the myocardium with a vascular network [**49-50**]. However, spontaneous differentiation of ESCs could lead to teratoma formation, which would be detrimental to myocardium cell therapy. To resolve this issue, cardiogenic cells could be separated from other differentiated cells to enhance the purity of cardiomyocytes and vascular cells [**51-52**]. Nevertheless, in the roadmap of ESC usage for clinical application, problems like non-autologous transplantation, immune rejection, fibrosis, arrhythmia [**53**] and ethical concerns must be considered.

To resolve the ethical concerns in the use of embryos for obtaining ESCs, induced pluripotent stem cells (iPSCs) were proposed as an alternative. Cardiomyocyte differentiation from mouse iPSCs was first reported in 2008 [**54-56**] and further studies showed that human iPSC-derived cardiomyocytes were similar to those from ESCs in terms of their expression of cardiac markers and their activity in action potentials [**57,58**]. Induced pluripotent technology opens up possibilities for autologous transplantation from patient's own cells. However, transformation of harvested adult cells to iPSCs requires stable transfection, which is currently most reliably performed via viral methods. Unfortunately, such viral transfection could also lead to possible karyotypic abormailities. Thus, such a strategy could undermine its clinical suitability. Non-viral transfection methods like transposons [**59**], protein- and mRNA-based delivery, [**60-62**] and the use of small molecules [**63**] have been developed to replace

retroviral vectors but are far less efficient. In addition, iPSCs alike ESCs have equal tendencies to form teratomas due to their likelihood for spontaneous differentiation.

In considering the range of possible cell sources, the quantity and quality of the final cell product must be considered. This means the capacity for cell expansion towards clinically relevant numbers, lineage specificity, and maturity of derived cardiomyocytes must be achievable from the selected cell source. Bioreactor design and cell culture parameters must be taken into consideration to ensure successful cardiac tissue engineering *in vitro* and its clinical relevance *in vivo*. Through a systematic approach in bioprocess engineering, the design of future bioreactors and integration of well-suited cell culture technology can ensure successful tissue engineering applications of specific cell types and lineages.

Bioreactor design and bioprocess considerations

Conventional methods for directing cardiomyogenesis *in vitro* are carried out in 2D flask/plate cultures, involving the use of TCP. Typically, cells are first expanded to reach the required cell numbers and then differentiated toward the cardiomyogenic lineage. The limited surface area on TCP restricts the cell densities achieved on 2D platforms, thus the expansion to clinically relevant cell numbers require the handling and usage of large number of TCP flasks. In the scale up of tissue engineering applications, bioreactors are frequently used with various possible configurations to meet specific applications and user preferences. Herein we provide a brief overview of different bioreactors that have been tested for both differentiation and maturation of cardiomyocytes.

One of the most commonly studied configurations is the spinner flask system [**6,7,52,64**]. To date, several companies are already providing commercially available configurations such as the Dasgip cellferm-pro system and Sartorius 2L Biostat MD. The basic spinner flask bioreactor design, as illustrated in **Figure 1a**, consists of an impeller, which provides a mixing action to ensure a well-mixed environment for cell culture. Mixing enhances homogeneity of the cell population
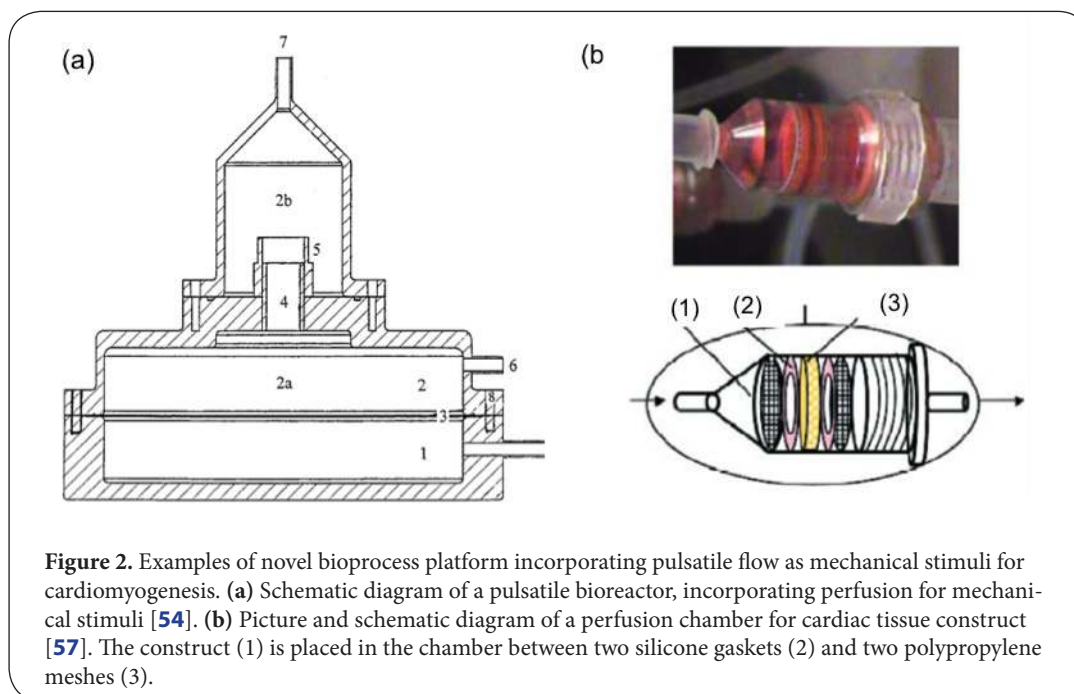
Figure 2. Examples of novel bioprocess platform incorporating pulsatile flow as mechanical stimuli for cardiomyogenesis. **(a)** Schematic diagram of a pulsatile bioreactor, incorporating perfusion for mechanical stimuli [**54**]. **(b)** Picture and schematic diagram of a perfusion chamber for cardiac tissue construct [**57**]. The construct (1) is placed in the chamber between two silicone gaskets (2) and two polypropylene meshes (3).

during proliferation, embryoid body (EB) formation (for ESC applications), and directed differentiation. Cells cultured in spinner flask bioreactors are either seeded on scaffolds or suspended as substrate-free aggregates [**7,52**]. The main advantages of this design are the ease in scaling up the bioreactor and the readily available monitoring capabilities. However, the impeller motion produces a shear stress environment that could lead to fatal cell damage and detrimental to cardiomyogenesis.

Another configuration that enables homogenous mixing without the use of impellers is the microgravity simulated rotating systems. A commercially available system is the rotary cell culture systems (RCCS) by Synthecon, which has also been studied for cardiomyogenesis [**65,66**]. This system was first designed by NASA to simulate cell culture under microgravity through rotation of the whole vessel. The rotating motion could provide homogeneous mixing under an ultra-low/no shear stress environment. The current commercial designs exist in two main configurations: the high-aspect rotating vessel (HARV, **Figure 1b**) and the slow-turning lateral vessel (STLV, **Figure 1c**). Both configurations are known to produce minimal shear stress and provide a suitable environment for cell cultures requiring low shear stress environment.

Besides the two commercial bioreactor designs highlighted, other novel bioreactors have been designed and studied by individual research groups. A notable feature in several lab-designed bioreactors is the use of pulsatile flow to introduce mechanical stimulation or shear stress to the cardiac constructs for directing cell differentiation or cell maturation [**67-71**]. For instance, Hoerstrup *et al.,* designed a bioreactor [**68**],

as illustrated in **Figure 2a**, where controlled, pulsed airflow introduced into the chamber (1) would cause an expansion of the silicone diaphragm (3). This expansion would then push the fluid though the channel (4) and cellular construct seeded in position (5), causing mechanical stimulation on the construct. Similarly, the pulsatile perfused bioreactor, designed by Brown *et al.,* [**71**] (illustrated in **Figure 2b**), produced construct with increased hypertrophy and a higher percentage of elongated cardiomyocytes. Besides the use of mechanical stimuli, electrical stimulation could also be incorporated into the bioreactor platform and this has shown to enhance cardiomyogenesis from sources like ESCs [**72-73**]. Both the electrical stimulation studies on ESCs found that the impact on cardiac differentiation due to electrical stimulation involved the generation of reactive oxygen species.

The oxygen level inside the bioreactor is an important factor that could affect cardiomyocyte yield. Several studies showed that hypoxia (4% oxygen level) affected cardiomyocyte differentiation of both human and murine ESCs; increased cardiomyocyte yield per murine ESC input [**52**] and increased expressions of cardiac markers in human ESCs [**7**] were observed, respectively. The benefits of hypoxia could be due to increased levels of hypoxia-induced factor (HIF-1α) which promotes cardiomyogenesis of ESCs [**74**]. However, hypoxia also aided in maintaining pluripotency and proliferation of ESCs, and resulted in decreased differentiation towards cardiomyocytes [**75**]. A more detailed review on the impact of oxygen on ESCs was discussed by Millman *et al.,* [**76**]. Although it has been observed that in general, hypoxia was preferred for cardiac differentiation from ESCs, culturing

neonatal cardiomyocytes in constructs at 20% oxygen level produced better contractile properties, cellular alignment and higher expression of sacromeric α-actinin [77-79]. Control of oxygen inside the bioreactor is essential in optimizing the yield and quality of cardiomyocyte generated from respective cell sources.

In designing a suitable bioreactor for cardiomyogenesis, a number of key design features must first be considered in the selection of a bioreactor configuration. These features include:

1. Culture conditions – Consider the feeding strategy, oxygen level, mechanical stimulation, etc.

2. Homogeneity of the culture environment – Consider the preferred mode of mixing operation to achieve the desired well-mixed environment.

3. Substrate for attachment – Consider whether the cells require a substrate for attachment to achieve 3D mimicry.

4. Scalability – Consider the factors necessary to design a scalable bioprocess.

These basic design questions serve to provide a guideline for one to consider the important features that should be integrated into the bioreactor design specified for cardiac tissue engineering applications. Distinct features of the bioreactor will impact characteristic parameters such as hydrodynamics. Thus, the selection of a bioreactor and execution of the bioprocess will result in different flow patterns affecting the shear environment and transport of soluble factors to the cell, which has a direct impact on cellular activities. For most tissue engineering applications including cardiomyogenesis, hydrodynamics in the bioreactor must be optimized to provide the suitable extracellular microenvironment for cell culture.

Hydrodynamics effects on cardiomyogenesis

Hydrodynamics is defined by fluid flows within the confined area of the cell culture platform. The static environment in conventional dish/flask cultures results in drastic concentration gradients and localized extremes that make large-scale bioprocesses difficult to monitor and control. Hydrodynamics in bioreactors can be optimized to alleviate such problems, making large-scale production easier. Different bioreactor configurations will result in different flow patterns that can affect cellular activities. In *ex vivo* generation of cardiomyocytes and 3D cardiac tissue constructs, hydrodynamics determines the macroscopic environmental conditions that will affect the shear stress and solute transport to the cells.

Simulation studies in hydrodynamics of different culture platforms can be modeled via computational fluid dynamics (CFD). The high efficiency in computational methods enables rapid interrogation of multiple parameters such as mass transfer efficacy, oxygen distribution and shear stress in various bioreactor platforms. Models from CFD simulations can generate relevant flow information to study the mass transfer efficiency of soluble factors to the cells. This not only identifies sensitive parameters that are significant to

the hydrodynamics of the bioreactor, but can also serve to guide in designing a bioreactor for optimal cell growth. Such stimulation studies have already been accomplished in airlift bioreactors and perfused bioreactors for bone and cartilage tissue engineering [80-84].

Hydrodynamics effects have not only been shown in CFD simulation models but have also been observed experimentally. For instance, expansion of both human HSCs and ESCs were higher and sustained for a longer period under mixed environment in stirred systems and rotating wall vessels than in the static cultures [66,85-87]. The improved cell expansion may be attributed to the improved transport of soluble factors. However, agitation speed in stirred systems must be optimized to produce a suitable shear stress environment for the different cell lines. This suitable environment should ensure maximum cellular viability, as observed for human HSCs [85], and optimal aggregation of EBs in the case of ESC cultures [66]. Evaluation of hydrodynamic effects on stem cell culture in general have been reviewed extensively by Kinney *et al.,* [88] and Vunjak-Novakovic *et al.,* [89]. Herein we will focus on the direct impact on cardiac tissue engineering applications and discuss potential implications in the bioreactor design.

Shear stress

Shear stress is introduced by the interaction of fluid molecules and cells due to fluid motion generated by mechanical movement of either the culture vessel or its components (such as rotary blades and flow perfusion). Differences in bioreactor configuration and their agitation will dictate the magnitude of shear stresses. Rotating wall vessels produce dynamic and laminar flow profiles that give rise to negligible shear stress [90-91]. Meanwhile, spinner flasks produce unidirectional and turbulent flow patterns that generate mild to considerable shear stresses. Rotational speed of the orbital-shaking or rotating wall vessel cultures will also affect the development of flow regions and magnitude of shear stresses. For instance, horizontal orbital-shaking vessels were found to produce uniform areas of low shear stresses of less than 2.5 dyn/cm^2 at an orbital speed of 20-60 rpm [92]. Meanwhile, optimal speeds in rotating wall vessels are able to achieve a microgravity environment while preventing any deleterious interaction with the wall boundary. This was found to be between 20-25 rpm for encapsulated murine ESCs [93].

Expansion and viability

Shear stresses pose a direct impact on cell viability and proliferation. It is therefore critical that the shear environment is optimized to ensure high proliferation and viability of the cell sources. Bioreactor configurations that could provide a well-mixed environment have generally shown to improve proliferation of human-derived cell sources like ESCs, MSCs and HSCs [91,94-95]. In a study involving human ESCs, mild shear stress environment in a spinner flask system with glass-ball impellers (IGB) and shaker flask systems resulted in higher

expansion fold and viability of ESCs than those observed in both the negligible shear environment in slow turning lateral vessels and the higher shear stress environment in spinner flask with paddle impellers. Other studies have also shown that shear stress higher than 2.5 dyn/cm^2 would cause cellular damage and reduce cell expansion; this is true for both human ESC and neonatal rat cardiomyocyte cultures [71,96,97]. In the case of rat BM-MSC expansion, proliferation would only decrease at higher levels under laminar shear stress conditions [98,99]; when BM-MSCs were exposed to 5 dyn/cm^2 of shear for 4 hours, proliferation decreased significantly [99]. However, shear conditions of 1 dyn/cm^2 did not affect cell proliferation, indicating that BM-MSCs could still be cultured under a mild shear environment.

To translate the shear value to a more obvious attribute, we evaluate the impact with respect to flow rates. It has been observed that fluid jets at velocities higher than 1.6 m/s would cause severe cell damage to attached cell layers of neonatal rat cardiomyocytes; causing them to detach from the substrate [100]. Conversely, a negligible shear environment in rotating wall vessels yield higher cell number and metabolic activity of these cardiomyocytes [90]. Rotary systems with insignificant shear environment produced laminar flow profiles, which serve to minimize cellular damage and increase aerobic metabolism, as compared to the unidirectional turbulent environment in spinner flasks. Laminar flow could also serve as a protective conditioning against apoptosis while turbulent flows would induce apoptosis [101].

Embryoid body formation

Embryoid body formation is considered a necessary step in the culture process of *in vitro* ESC differentiation. Similar to culture practices in traditional flask cultures, one main challenge in executing ESC differentiation in a bioreactor is the control of EB formation. However, unlike the static conditions maintained in most flask cultures, hydrodynamics and the impact of shear stresses in dynamic systems would play an important role in controlling EB formation. The next few studies have shown how shear stress would affect EB formation from human-derived ESC. Yirme *et al.,* has demonstrated that the initiation of EB formation was highly sensitive to shear stresses, when human ESCs are directly seeded into the dynamic culture system under a shear environment, low EB concentration would result [91]. Thus a 2-day EB prearrangement period in static culture was recommended prior to seeding the cells into dynamic bioreactor systems. Subsequent to the prearrangement period, control of EB concentration, shape and size distribution was found to be more conducive under mild shear forces. Two stirred bioreactors (Erlenmeyer and IGB spinner flask) that were compared to Petri dish and STLV bioreactor cultures showed that mild shear stress in the dynamic culture systems produced smaller but more homogeneous EBs [91]. Another study also showed that the most uniform propagation of the human EB population

can be achieved under conditions below 2.5 dyn/cm^2 [96] forming a homogenous EB population. Homogeneity of EB populations appeared to follow an increasing trend with the speed of mixing as long as the shear stress was kept under this operational limit [91,92].

Cardiomyogenesis

The cardiac differentiation potential of human ESCs could be enhanced by mild shear stresses in the IGB spinner flask and the Erlenmeyer flask [91]. Increased number of contracting EBs (at least 30%) were found in the spinner flask cultures and higher cardiac specific transcripts levels were observed in both spinner and Erlenmeyer flask cultures. Such observations were in agreement with those made by Sargent *et al.,* in their human ESC studies, in which cardiac gene markers had the highest up-regulation in an environment with shear stress less than 2.5 dyn/cm^2 [92]. For BM-MSCs, the highest levels of cardiac markers such as Nkx2.5, β-MHC and ANP were expressed when the rat BM-MSCs were first conditioned by a shear level of 10 dyn/cm^2 for 24 hours in a parallel-plate flow chamber prior to differentiation. Under this shear condition, the microfilaments produced were the thickest and most well-oriented amongst all other experimental groups [102]. The impact of laminar shear stresses on BM-MSCs were also noted; any shear stress levels above 5 dyn/cm^2 would inhibit rat BM-MSC proliferation [99]. The inhibition of cell proliferation in this study was associated to cell cycle arrest [99] while another study showed that flow induced shear stresses could simulate specific differentiation towards osteogenesis [103]. From the BM-MSCs studies, we can infer that shear stress could cause a phenotypic change in BM-MSCs. When the cells are subjected to a substantial level of shear stress, BM-MSCs could switch from proliferation to differentiation, leading to lineage specificity. Therefore, shear stress could have beneficial effects on cardiomyogenesis of BM-MSCs. When comparing the shear stress tolerance of ESCs and BM-MSCs, the tolerance of BM-MSCs appears to be higher than that of ESCs. This could be due to two reasons. First, the mentioned ESCs are derived from human while BM-MSCs are of rat origin. Second, the tolerance level of shear stress may also depend on exposure time. The higher optimal shear stress required for BM-MSCs could be due to the fact that BM-MSCs were subjected to a transient shear environment for only 24 hours while ESCs were subjected to a continuous shear environment for several days. In general, the overall benefits of mild shear environments on expansion and differentiation of both embryonic and adult stem cells suggest that mild shear stress would be preferred for cardiomyogenesis.

In the maturation of cardiomyocytes, intricate tissue structure and contractile properties become primary indicators in evaluating the success of an engineered cardiac tissue. Numerous studies were conducted to determine whether shear stress could affect and improve the ultrastructural and electrophysiological properties of cardiac constructs. For

instance, a shear environment of 0.6 dyn/cm^2 could increase the expression of cardiac markers like sarcomeric α-actin, cardiac troponin-T, Connexin-43 and adhesion molecule, N-cad in encapsulated neonatal rat cardiomyocytes [97]. These constructs also produced cardiac muscle tissue with massive striations and well-developed sacromeres, and had regions where construct thickness exceeded 500 µm. This was in stark contrast to the aggregates of round cardiomyocytes in static and shear-free environment. Also, when cardiac constructs derived from neonatal rat cardiomyocytes were subjected to physiological shear levels of the vascular valves (1.54 dyn/cm^2) as imposed by a pulsatile flow, they exhibited better contractile properties like lower excitation threshold, higher maximum capture rate and higher contractile amplitude than those experiencing no shear or lower flow rates [71]. As shear stress was increased, electrophysiological properties such as the induction of extrasystoles also improved notably [100]. It has been suggested that the beneficial effects of shear stress is due to mechanical stimulation provided by the fluid movements and interactions, which induced subcellular changes, triggering action potential and mechanosensitive currents in maturing cardiomyocytes [100].

From these observations, controlled shear stress environments can serve as a mechanical stimulus to enhance maturation of cardiomyocytes. However, shear forces should not exceed certain shear stress limits. For example, studies have shown that shear environment above 1.6 dyn/cm^2 would cause immature cardiomyocytes in the constructs to adopt a rounded morphology instead of forming striations [104,105]. One possible argument for their preference to low shear stress conditions could be derived from the *in vivo* situation. *In vivo,* cardiomyocytes are mainly found in the myocardium, which is between the inner lining endocardium and the epicardium. The endocardium consists primarily of endothelial cells. It serves as a buffer layer in the exchange of solutes to the myocardium and as a protective layer that shield the cardiomyocytes from blood flow during the pumping of ventricle chambers. Thus, when cardiomyocytes were subjected to high shear stress, they would show signs of dedifferentiation and adopt a rounded morphology.

Collectively controlled shear stress under mild conditions would enhance cardiomyogenesis for cardiac tissue engineering. To date, experimental studies suggested that the upper limits for induction of cardiomyogenesis from adult or embryonic stem cells would be 2.5 dyn/cm^2 and maturation of neonatal cardiac cells would be 1.6 dyn/cm^2. In designing a bioreactor, operational limits should not exceed the upper limits found in these experimental studies and for long-term cultures, the operational range should be even more conservative given the heightened sensitivity of the cells over a long period of exposure to shear stress.
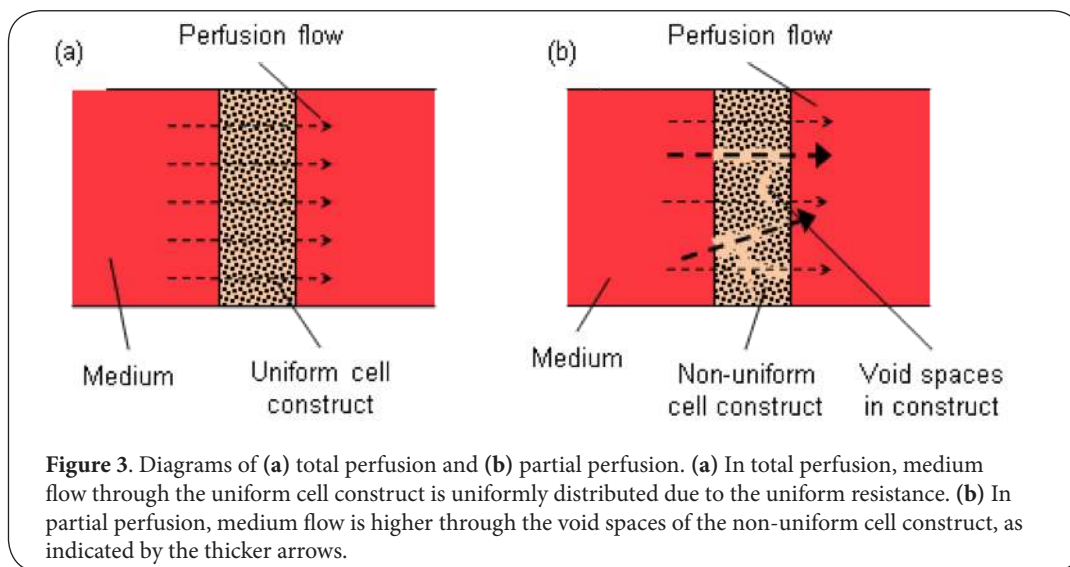
Mass transport
Hydrodynamic flow affects the transport within a culture vessel and controls the availability of nutrients and soluble factors to the cells. For a static culture, transport of soluble components in the culture medium is mainly via diffusion. The lack of fluid flow in a static culture will result in concentration gradients within the bulk fluid, causing localized extreme conditions and posing several challenges in the control and monitoring of large-scale cell production. One of the main strategies to minimize non-homogeneous cell culture environments is to achieve uniform mixing in the culture vessel by introducing convective transport.

Bulk convective transport
In spinner flasks and rotating wall bioreactors, bulk fluid transport is facilitated via convection and provides a more homogenous environment throughout the vessel. Cultures would generally produce higher cellular expansion and better cell viability in such environments due to more efficient transport of soluble components, which supports cell activity [89,93]. Contractile properties of the cardiomyocytes along the peripheral surface of cardiac constructs were more homogeneous in a well-mixed environment [106]. Besides the higher cell number, better cell viability, and improved homogeneity, cultures in a well-mixed environment result in enhanced cardiomyogenesis. For instance, murine ESCs were found to express higher levels of cardiac markers like Nkx2.5, α-MHC and ANF in rotating wall vessels than in static cultures [65,93].

Although mixing is beneficial for the cell culture, flow patterns inside the vessel are affected by the mode of mixing and this will impact the convective transport efficacy. Shaker flasks were found to have a lower mass transfer coefficient than spinner flasks and therefore would not be suitable for cell cultures requiring high oxygen demand such as during maturation of cardiomyocytes [107]. Agitation speed must also be adjusted to minimize concentration gradients and shear stresses that may be deleterious to the cells. In a comparative study between different culture systems, the rotating wall vessel had produced neonatal rat cardiomyocytes constructs of higher viability and higher expression levels of Connexin-43 and creatin kinase-MM than the spinner flasks [106]. Even though both bioreactors provided homogenous environments, the higher shear stress in spinner flask is detrimental to cardiomyogenesis. For rotating wall systems, the optimal rotation speed must achieve microgravity and maintain cellular constructs within the mid zone area to minimize collison to the wall boundary of the bioreactor. Experimental studies have confirmed that the rotation speed of the HARV rotating wall vessel system must operate above 25 rpm, when culturing hydrogel-encapsulated murine ESCs, in order to ensure uniform oxygen concentration above the

Figure 3. Diagrams of **(a)** total perfusion and **(b)** partial perfusion. **(a)** In total perfusion, medium flow through the uniform cell construct is uniformly distributed due to the uniform resistance. **(b)** In partial perfusion, medium flow is higher through the void spaces of the non-uniform cell construct, as indicated by the thicker arrows.

viable threshold [93]. Meanwhile cardiac constructs seeded in 600 μm microbeads required a speed of 18 rpm [**108**].

Solute transport within 3D cellular construct is governed by molecular diffusion. The transport efficacies within these constructs will therefore have a direct impact on the cell's microenvironment. Diffusion limitations can result in heterogeneous cell distribution and/or compromise cell viability in regions distant from the periphery of the construct. Cardiac constructs in both rotating wall vessels and spinner flasks were found to sustain a viable cellular region of up to 100 μm in thickness along the peripheral region, while having an acellular core containing mostly apoptotic/dead cells. The latter is a consequence of diffusion constraints in oxygen [**90,109**]. A simple mass transfer simulation could be used to estimate the availability of nutrients inside 3D constructs and can be modeled based on diffusion and consumption rates of oxygen and relevant metabolites. With reference to a hepatocyte spheroids model [**110-111**], an increase in spheroid size would increase the core area, which would experience oxygen levels below the critical survival threshold. The critical radius of such spheroids could be calculated from the following:

$$r = \sqrt{\frac{6DH(P_{O2} - P^*)}{O2 \text{ consumption rate}}} \ldots\ldots\ldots (1)$$

where   D: Diffusion coefficient of oxygen within aggregate
   H: Henry constant of oxygen from air to medium
   P_{O2}: Partial pressure of oxygen in the air
   P*: Critical pressure of oxygen for cell survival

Although oxygen transport face diffusion constraints, the transport of glucose within a 3D construct is less affected by the spheroid size and its distance from the peripheral surface. This is due to the fact that the effective diffusivity of glucose was not as significantly affected by the cell metabolic rate as that of oxygen. In general, effective transport throughout a 3D cellular construct requires the optimization of both convective and diffusive transport through careful selection of the bioreactor and design of the supporting scaffold.

Perfusion

Perfusion enables a continuous supply and control of culture parameters like pH, oxygen, nutrients, and metabolites to maintain optimal operating conditions throughout the culture period. Two classifications have been defined: total perfusion and partial perfusion [**112**]. Total perfusion occurs when the flow is directed through a homogeneously and densely packed construct, which occupies the entire cross-sectional area of the perfused flow (illustrated in **Figure 3a**). Partial perfusion occurs in a non-homogeneously packed construct where channels are present or when constructs are freely suspended in culture (represented in **Figure 3b**). Both perfusion modes will result in different transport profiles in the construct and therefore will have different impact on cell numbers and cardiac differentiation.

Perfused cultures generally produced higher cell numbers than static cultures and were able to achieve expansion efficacy equivalent to the spinner flask cultures [**104**]. Furthermore, perfusion can be used to modulate oxygen availability to produce more uniform cell distribution throughout the cardiac construct and achieve more aerobic metabolism than both static and spinner flask cultures [**89,104,113**]. Perfusion rates tuned within operable conditions 0.2-3.0 mL/min can control the availability of oxygen to cardiac cells inside the constructs [**77**]. However, transport within the construct between total perfusion and partial perfusion could differ drastically [**112**]. For total perfusion, cell density and viability are compromised

in the regions that are further from the construct surfaces due to lower oxygen levels. Oxygen levels in the bulk medium could be increased to alleviate this problem, and thus provide an oxygen level above the critical survival threshold to the deeper regions. Under partial perfusion, regions with lower cell density will present lower resistance to the flow of medium. Thus medium would prefer to flow through such regions of lower resistance and result in significantly higher flow rates. When the percentage of low-resistant regions is insignificant, the construct would have a uniform resistance throughout the construct volume, and this would be equivalent to a cell distribution and transport profile to that in total perfusion. However, as the percentage of the low-resistant regions increases, cells will be more densely distributed along the low-resistant peripheral surfaces, which are exposed to the flow. Under such a scenario, diffusion within the construct would be more dominant than convection, resulting in similar phenomena as in the non-perfused cases.

Beneficial effects of perfusion on the extent of cardiac differentiation were also observed. Perfused cultures could produce cardiac constructs, from neonatal rat cardiomyocytes, which are more uniformly distributed throughout the construct [104] and have better contractile properties like twitch force and contraction frequencies than non-perfused cultures [79,114]. The role of perfusion in providing mechanical stimuli through shear stress was also demonstrated in a perfusion study through a series of constructs [77]. Their results showed that perfusion not only enhanced the induction of cardiomyogenesis, the shear stress from the perfused flow also enhanced the distribution and maturation of cardiomyocytes. In a recent study conducted by our group (unpublished data), the encapsulated murine ESCs in our novel perfused bioreactor produced earlier and higher cardiac expressions like cardiac Troponin-T and α-MHC upon differentiation, in comparison to the non-perfused cultures. Results from the reviewed studies showed that perfusion enhances homogeneity within the constructs and helps in maturation of cardiomyocytes. Meanwhile, the results from our group showed an earlier and higher induction of cardiomyogenesis in murine ESCs. These results were complementary, showing the overall benefit of incorporating perfusion into bioreactors for cardiac tissue engineering.

Contributions in hydrodynamics as shown by shear stresses and transport play a significant role in the success of cardiac tissue engineering in both macro and micro-scale. The availability of vital nutrients and oxygen is important in supporting overall cell viability and can impact the extent of differentiation. The design of dynamic bioreactors for 3D cardiac tissue engineering applications must therefore take the effects of hydrodynamics into careful consideration and the observations that have been made, highlighted the impact of hydrodynamics. These are summarized in **Table 1**.

Scaffold architecture

Use of substrate
To facilitate culture in scalable and dynamic bioreactors, cardiac cells and progenitor cells require a substrate for cell attachment. 3D constructs are preferred as higher cell densities per vessel volume can be achieved [115] and these constructs could provide a better mimicry to the *in vivo* state. For instance, ESC cultures in 3D can be achieved via the formation of embryoid bodies, cell seeding in a 3D scaffold or cell encapsulation in a hydrogel. The choice of substrate material and physical properties of the scaffold design would impact hydrodynamics in the micro-scale.

For cardiomyogenesis involving human and murine ESCs, many studies have directed cardiomyogenesis through the formation of embryoid bodies without attachment on any substrate material [6,66,116-120]. However, cellular products that are derived from the substrate-free method are highly dependent on operational parameters such as rotation and shear environment. This results in large variations in the cellular aggregate population, and imposes difficulty in controlling cell differentiation and maturation. Alternatively, many dynamic/suspension bioreactor cultures are conducted using microcarriers [5,96] where the control of cell spheres and process scalability can be more readily achieved. The third possibility is to perform cell encapsulation of adult stem cells, ESCs, and cardiomyocytes [121-126]. Hydrogels, such as Ca-alginate, could be used to control the aggregate size during proliferation and showed to support long-term human ESC culture [126-128]. Moreover, encapsulated cells are less exposed to the shear environment. As reviewed in the previous section, studies on shear stresses have concluded that directing cells toward cardiomyogenesis in a low shear stress environment is generally more advantageous; cell encapsulation can also help in reducing shear stresses.

The selection of material as a suitable substrate for tissue engineering is the first important consideration. In general, the material should be biocompatible and sterilisable. In some instances, the mechanical properties of the materials must also match with those of the host tissue, and biodegradability if required should not exude cytotoxic components. There are two main categories of polymers that can be used for synthesis: naturally derived polymers and synthetic polymers. Substrates made from naturally derived polymers include alginate and collagen [129-132] which is more favorable for cell attachment. However, their mechanical properties can be more difficult to control and the inter-batch variability is subjective to their derived sources. Synthetic polymers like poly(ethylene glycol), poly(lactic acid) (PLA) and phosphoester, enable better tunablility of mechanical properties by changing parameters of the synthesis process. However, synthetic substrates generally do not promote cell adhesion as efficiently as those from naturally derived polymers. In such cases, extracellular matrix (ECM) derived peptides and proteins like Arg-Gly-Asp (RGD), gelatin and laminin, can be incorporated on the synthetic polymer chains to enable better cell adhesion and enhanced

Table 1. Summary of hydrodynamic effects on cellular activities (cell proliferation and distribution and cardiomyogenesis).

Cell proliferation and distribution				
	Shear level			
	Negligible	Mild	Significant (Eg ESC: >2.5dyn/cm2 BM-MSC: >5dyn/cm2)	
Expansion fold	+	++	-	Yirme *et al* 2008, Lecina *et al* 2010, Dvir *et al* 2007 Kreke *et al* 2004 Luo *et al* 2011
	Transport mode			
	Diffusive	Convective	Perfusion	
Expansion and viability	-	+	NA	Consolo *et al* 2011
Uniformity and metabolism	-	+	++	Carrier *et al* 2002, Radisic *et al* 2007
Cardiomyogenesis				
	Shear level			
	Negligible	Mild	Significant (Eg ESC: >2.5dyn/cm2 BM-MSC: >10dyn/cm2)	
Differentiation to cardiomyocytes	+	++	-	Yirme *et al* 2008, Sargent *et al* 2010 Huang *et al* 2010
Cardiomyocyte maturation	+	+	-	Brown *et al* 2008, Dvir *et al* 2007, Carrier *et al* 2002, Radisic *et al* 2007
Cellularity and metabolism	+	-	-	Carrier *et al* 1999
	Transport mode			
	Diffusive	Convective	Perfusion	
ESC differentiation to cardiomyocytes	-	+	NA	Sargent *at al* 2009, Consolo *et al* 2011
Contractile properties	-	-	+	Hecker *et al* 2009, Radisic *et al* 2004

Negative sign ('-') indicates relatively negative impact; positive sign ('+') indicates moderate positive impact and double positive ('++') indicates significant positive impact

cardiomyogenesis [133-136]. In utilizing synthetic polymers, both the physical anitropsy and mechanical properties of the polymer scaffold can be tuned to desired features to promote not only cell attachment but also cell alignment [121,122]. The use of RGD peptides has also proven to be very effective in improving cardiomyocyte viability and enhancing contractile performance of differentiated cells [124].

In selecting a suitable substrate, one of the major considerations is the substrate architecture, which plays a significant role in the transport efficacy of soluble factors. The overall architecture can be controlled by different fabrication techniques. For instance, a study was conducted to compare the impact of scaffold fabrication on the maturation of cardiomyocytes. Cells were seeded on scaffolds synthesized by two different techniques: thermally induced phase separation (TIPS) and electrospinning [137]. The TIPS method involves cooling of a polymer-solvent mixture into polymer-poor and polymer-rich phases, followed by extraction of the solvent (typically through sublimation) to give rise to a porous scaffold. In electrospinning, an applied high voltage will introduce charge-repulsion onto the polymer solution and spins the solution into a thin fluid jet onto a collector plate. As the solvent evaporates, it would leave behind a random network of nanofibres. Electrospun scaffolds have a larger surface area to volume ratio and are usually thinner than TIPS scaffolds. In this study, murine ESC-derived cardiomyocytes seeded on TIPS scaffolds were densely packed along the outer periphery about 100-200 μm in thickness while cells seeded on thinner electrospun nanofiber scaffolds had a more uniform cellular distribution [137]. Though expressions of cardiac markers (sacromeric-MHC and comnexin-43) were similar in both scaffolds, cardiomyocytes on TIPS scaffold had round morphology while electrospun scaffold gave rise to elongated cells. This indicated that the TIPS scaffold gave rise to a more embryonic phenotype while the architecture of electrospun scaffolds is preferential for cell maturation of ESC-dervied cardiomyocytes.

Scaffold fabrication techniques determine the overall architecture of the material and its properties such as topography, porosity and even stiffness. The availability of soluble factors surrounding the cell's microenvironment is largely controlled by porosity of the scaffold. This can impact cell function such as proliferation and differentiation, determining the fate in cardiomyogenesis. Other parameters such as surface topography and stiffness can also impact cell signalling mechanisms via cell-surface interactions. **Table 2** summarizes the impact of these design parameters on cardiac tissue engineering and these examples are further

Table 2. Summary of effects of substrate architecture on cell proliferation and distribution and cardiomyogenesis

|		
Cell proliferation and distribution		
Porosity	Enhanced oxygen transport for porous scaffolds with 100um channels and spacings	Radisic *et al* 2007
Topography	Higher expansion fold and viability of neonatal cardiomyocytes on flat surface than on mesh network due to higher specific cell concentration per area	Shah *et al* 2010
Stiffness	Higher expansion fold and uniformity in substrate of less than 100kPa (low-medium stiffness)	Radisic *et al* 2004, Kenar *et al* 2011
Cardiomyogenesis		
Porosity	Higher purity of cardiomyocytes from other stem-cell derived products in 60um-channelled scaffolds with 20-40um pore network	Madden *et al* 2010
	Shift of immunoresponse to prohealing phenotype during transplantation in scaffolds with 30-40um pores	Madden *et al* 2010
	Better cardiomyocyte alignment during transplantation with increased porosity	Ifkovits *et al* 2010
Topography	Increased alignment and contractile properties along grooved and channelled surfaces	Aubin *et al* 2010, Fujita *et al* 2006, Yin *et al* 2004
	Optimised width and spacing of patterns required to allow suitable time for cell proliferation and alignment	Aubin *et al* 2010
Stiffness	Higher contraction amplitude and more beating areas on substrate of low stiffness	Shapira-Schweitzer *et al* 2007
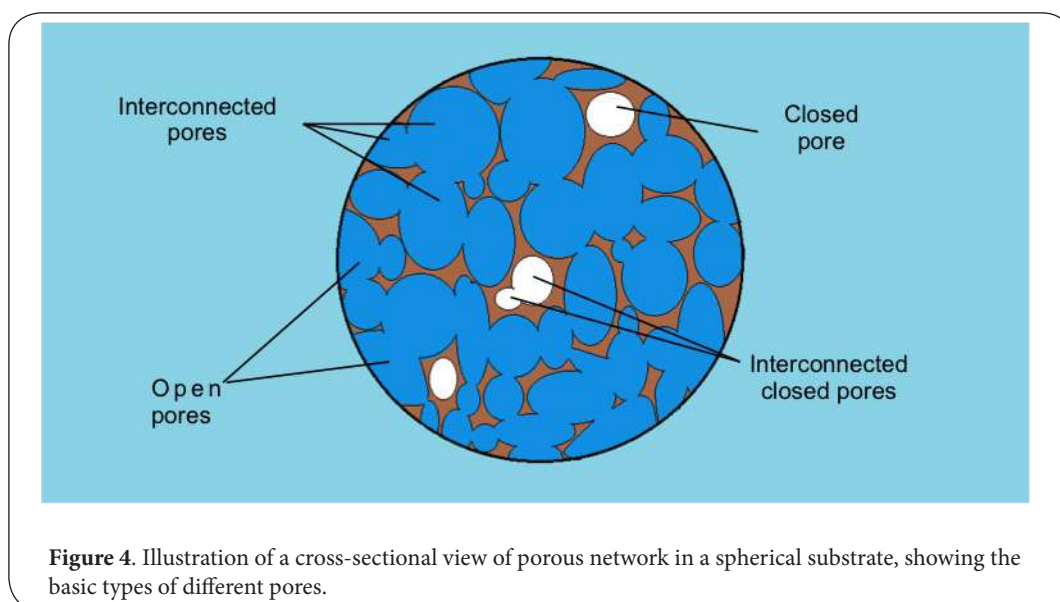
Figure 4. Illustration of a cross-sectional view of porous network in a spherical substrate, showing the basic types of different pores.

elaborated in the following sections.

Porosity

Porosity is a measure of void spaces in the scaffold and is a key design component in influencing the transport of soluble factors. In quantifying porosity of a scaffold, the percentage of void spaces and the level of interconnectivity, as illustrated in **Figure 4**, must both be considered. For tissue engineering applications, scaffolds with high interconnected porosity are preferred to minimize transport resistance and to promote cellular interaction and vascularization. In such highly interconnected and porous scaffolds, pore sizes and shapes could be varied to manipulate fluid transport and other cellular activities, as demonstrated by a number of studies.

Mesh scaffolds are usually random networks of pores synthesized by techniques like TIPS. Oxygen transport in random porous networks tends to have a higher degree of tortuisity than organized structures. Oxygen transport limitation in the mesh scaffolds therefore prohibits the formation of cardiac patches beyond 0.15 cm, even with the aid of oxygen carriers. On the other hand, channelled scaffolds enabled oxygen supply throughout the whole construct with thickness up to 0.5 cm when oxygen carriers were used. A mathematical model was developed to show how oxygen distribution in the parallel channel array can be optimized by varying scaffold geometry [105].

Besides enhancing transport properties, pore size and channel spacing can be used to promote cardiomyocyte bundle orientation *in vitro* and enhance neovascularization *in vivo*. Madden *et al.,* showed that a minimum spacing of 60 μm channels was necessary for human ESC-derived cardiomyocyte cell seeding and the promotion of cardiomyocyte aggregate formation [138]. Interconnectivity of a porous network could also enhance purity of the human ESC-derived cardiomyocyte population by permitting non-cardiomyocyte cells to migrate through the network, which was optimized by channel walls with 20-40 μm pores.

Furthermore, porosity could be used to control immunoresponse and cellular alignment *in vivo*. Scaffolds with 30-40 μm pores produced maximum vascularization and minimal fibrous cap formation in an *in vivo* study [138]. In the same study, a shift of macrophage phenotype from proinflammatory M1 to prohealing M2 was also observed in 30-40 μm pores. Such a shift would reduce the chances of *in vivo* rejection of implanted scaffolds for cardiomyogenesis. Porosity could also increase the rate of cellular infiltration and ECM deposition thus producing better aligned cardiomyocytes post-transplantation [139]. This effect would likely be more pronounced when using a scaffold material that has a slower degradation rate.

Topography

Topography refers to the surface features of a material. In a substrate for cell culture, topography defines the surface patterns in which cells come in direct contact with. Numerous studies have reported the effects of topography on cardiomyocytes, enabling cell alignment and enhanced cardiomyocyte maturation. Grooved or channeled surfaces and an anisotropic geometry of scaffolds were shown to enhance the alignment and contractile properties of cardiomyocytes [140-143]. For instance, better cell alignment of neonatal rat cardiomyocytes were observed in accordion-like honeycomb scaffolds than in isotropc controls [141]. Abrasions on polyvinyl surfaces at 13 μm wide and 700 nm deep were also found to

be optimal for neonatal rat cardiomyoctye elongation [**144**]. Both studies support the fact that cell alignment, orientation and elongation, can be readily achieved by contact guidance and will be useful in cardiac tissue engineering.

Substrate topography can also influence cardiomyocyte properties. The effect of microtopography was studied using acellular porcine urinary bladder scaffold [**145**], which consisted of two distinct surfaces: a luminal side with smooth surface texture and an abluminal side with fine mesh network of nano- and microfibers. The results showed that the flat luminal surface had a higher density of neonatal cardiomyocytes with better contractile properties than the abuluminal surface. Contractile properties such as calcium recovery and hysteresis of cells grown on the luminal side also had superior performance. Hysteresis is a short-term cardiac memory of the previous contractile patterns, and is indicative of electric stability. Its presence in the cells is believed to help in preventing arrhythmias during transplantation.

Microarchitectures were successfully created in 3D hydrogels to control cell alignment recently [**140**]. The micropatterned hydrogels facilitated self-organization and elongation of fibroblast, myoblasts and cardiac progenitor cells to form aligned microconstructs. As the width of pattern channels decrease, the percentage of cells aligned along the direction of the axis would increase. When substrates with patterned surface were used, seeding density could also be optimized as higher seeding density would require shorter time for alignment of cells along the whole channel. These topographical effects were observed only for directionally orientated cells, but not for non-directionally orientated adherent cells like hepatoma cells [**140**]. Overall, 3D micropatterning techniques will become highly beneficial for the construction of cardiac tissue constructs.

Stiffness

Stiffness is the rigidity of a material and thus its ability to resist deformation when a force is applied. In tissue engineering, substrate stiffness could affect cellular proliferation and cellular activities like mineralization in osteogenesis and contraction in cardiomyogenesis. Substrate stiffness is usually controlled by concentrations of reagents such as the precursor or the monomer itself. Besides varying the material stiffness, concentration of the reagents could also affect the density of biologically active motifs in the substrate which will affect the cellular contractile properties [**146**].

On a substrate of low or medium stiffness (<100kPa), such as a 2% PGA scaffold, higher cardiomyocyte proliferation and a more uniform cell distribution were observed than that of a stiffer scaffold [**115,135**]. In addition, stiffer substrate was also found to be detrimental to cardiomyocyte maturation. In a study using PGS scaffolds and PEGlyated fibrinogen hydrogels to culture neonatal rat cardiomyocytes, lower contraction amplitude and less beating areas were observed in the stiffer materials [**146**]. Possible explanations on the detrimental effects
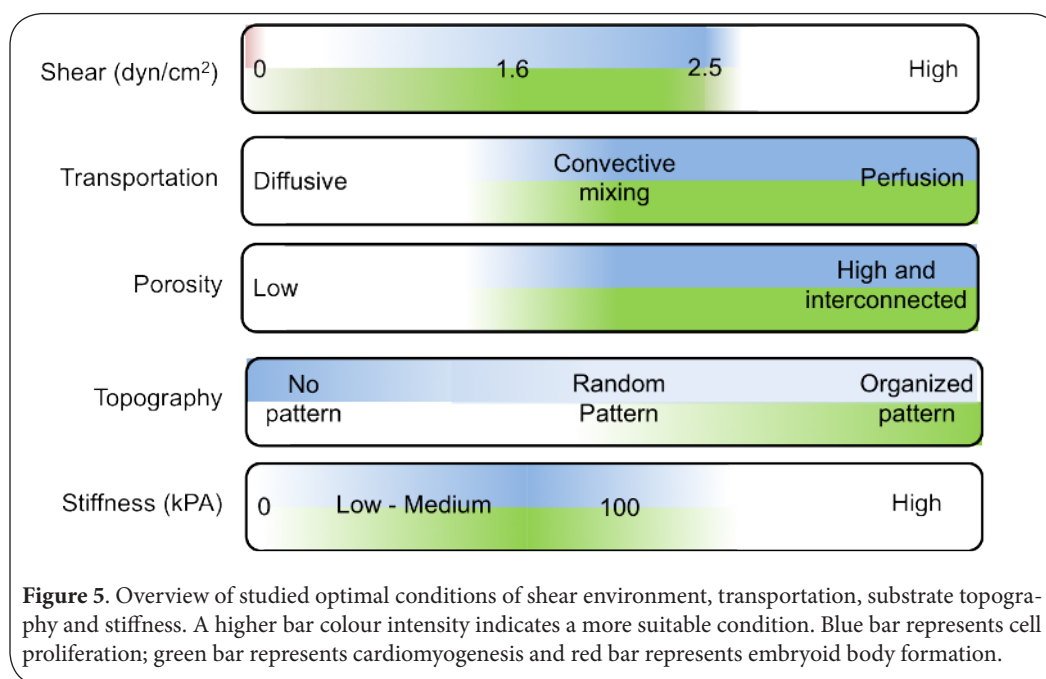
of stiffer substrates could be that (1) stiffer substrates affected the contractile mechanism or (2) lower cell numbers and less uniform cell distribution in the stiffer scaffolds would yield lower cell-cell interaction, and this effectively inhibited cell maturation. Typically, substrates with a low stiffness would be more suitable for cardiac tissue engineering applications in promoting cell proliferation, uniformity of distribution and maturation. Moreover, substrates of lower stiffness would permit more flexibility in the construction of a myocardial patch; as in the case of a flexible microfibrous scaffold, which would be easier to be rolled into a tubular form than one with a higher stiffness [**115**].

Conclusion and future directions

In generating cardiomyocytes with clinically-relevant numbers, large-scale dynamic bioreactors will play an essential role in achieving the greatest potential. The control of hydrodynamic conditions in the macro- and microenvironment, tailored to cardiomyogenesis *in vitro,* is vital for optimizing the quality and reproducibility of engineered cell-tissue construct. Microarchitectural design of the substrate also plays a part in cell and tissue organization, which affects cell function. We summarized different parameters that could be used to provide optimal conditions for cell proliferation, embryoid body formation and cardiomyogenesis (**Figure 5**). The summary figure tells us that optimal shear conditions for cell proliferation and cardiomyogenesis should be generally mild and that perfusion is preferred in both proliferation and differentiation processes to facilitate expansion, homogeneity and maturation. In terms of substrate architecture, highly porous and interconnected scaffolds that are of low stiffness (<100 kPa) are favored for both cell proliferation and differentiation. In addition, an organized topography would only play a role in cardiac differentiation.

The summary of these findings can serve as an excellent guideline in the selection and design of bioreactor cultures for cardiac cell tissue engineering. Depending on the cell source, this is the first step in optimizing the conditions to yield the required cellular products. However, control of culture conditions is imperative to large-scale manufacturing of engineered cells and tissues. The ability to incorporate in-situ monitoring and control measures is an essential component in large-scale production processes to ensure reproducibility. Technologies with such purposes have been established in the industries to guarantee product quality and uniformity and satisfy GMP requirements. At the current stage, large-scale cultures for cardiac tissue engineering are still in the midst of establishing suitable bioreactor platforms and the monitoring and control technologies have not yet been fully-incorporated into these designs. With process analytical technology (PAT) initiative [**147**], real-time monitoring and control are now recommended and such technologies would become indispensible in the near future.

To date, many real-time monitoring methods are available for

Figure 5. Overview of studied optimal conditions of shear environment, transportation, substrate topography and stiffness. A higher bar colour intensity indicates a more suitable condition. Blue bar represents cell proliferation; green bar represents cardiomyogenesis and red bar represents embryoid body formation.

bioreactors, including conventional electrochemical sensors, and non-invasive spectroscopic and optical technologies [148,149]. Integration of real-time monitoring technologies should minimize the amount of sample extracted for analysis and any disruption to the cell culture itself. Alternatively *in-situ* probes may be incorporated into the bioreactor and this is one of the most favored designs. However, *in-situ* methods face the challenges of maintaining component sterility and its incorporation into the sterile environment of the bioreactor. The design of a minimally invasive yet reliable monitoring technique remains as one of the main challenges to tissue engineering.

In reviewing the achievements and progress over the past decade, we have moved closer toward a better understanding of the needs and requirements in cardiac tissue engineering and have developed many tools and techniques that can enable us to achieve 3D functional cardiac cell tissue constructs. Despite improved understanding and new findings, functionally viable tissue-engineered cardiac constructs that can be used to repair/replace damaged heart tissues remain a significant challenge. Firstly, the choice of cell source for cardiac tissue engineering each faces different challenges that need to be addressed independently. For example, use of pluripotent stem cell resources will need to ensure homogeneity and purity of cardiomyocytes prior to transplantation. Next, transport limitations constrain the size of tissue engineered constructs and calls for the need of a vascularized network. Parameters required for tissue integration of cardiac cell patches that will enhance overall cardiac function is highly multifactorial and not easy to achieve.

List of abbreviations
ASC: Adipose-derived stem cell
BM-MSC: Bone marrow stem cell
CAP: Cardiac-derived adherent proliferating
CFD: Computational fluid dynamics
CSC: Cardiac stem cell
CPC: Cardiac progenitor cell
CVD: Cardiovascular diseases
EB: embryoid body
ECM: Extracellular matrix
ESC: Embryonic stem cell
HARV: High-aspect rotating vessel
HSC: Hematopoietic stem cell
IGB: Glass-ball impellers
iPSC: Induced pluripotent stem cell
MSC: Mesenchymal stem cell
PAT: Process analytical technology
RCCS: Rotary cell culture systems
STLV: Slow-turning lateral vessel
TCP: Tissue culture polystyrene
TIPS: Thermally induced phase separation

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