A Platform for High-throughput Mechanobiological Stimulation of Engineered Microtissues

by

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Department of Mechanical and Industrial Engineering
University of Toronto

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2012

Abstract

While tissue-engineering approaches of heart valves have made great strides towards creating functional tissues in vitro, the instruments used, named bioreactors, cannot efficiently integrate multiple stimuli to accurately emulate the physiological microenvironment. To address this, we conceptually designed and built a bioreactor system that applied a range of mechanical tension conditions, modulated matrix stiffness, and introduced biochemical signals in a combinatorial and high-throughput manner. Proof-of-concept experiments on PAVIC-seeded hydrogels were performed to assess the independent and combined effects of tensile strain, matrix stiffness and TGF-β1 on myofibroblast differentiation by measuring α-SMA expression, a marker that indicates a disease-associated phenotype. We found that matrix stiffness and TGF-β1 significantly increased α-SMA levels (p < 0.001), while the effect of mechanical strain was only significant on soft gels (~12 kPa) without TGF-β1. This study therefore demonstrated independent and integrated effects of multiple stimuli in regulating key cellular events in the aortic valve.
Acknowledgments

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AVD</td>
<td>Aortic valve disease</td>
</tr>
<tr>
<td>BioLP™</td>
<td>Biological laser printing (trademark)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAVD</td>
<td>Calcific aortic valve disease</td>
</tr>
<tr>
<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
</tr>
<tr>
<td>CVD</td>
<td>Calcific valve disease</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FEA</td>
<td>Finite element analysis</td>
</tr>
<tr>
<td>gelMA</td>
<td>Gelatin methacrylate</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropanol</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency-associated protein</td>
</tr>
<tr>
<td>MMI</td>
<td>Man-machine interface</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>OPN</td>
<td>Osteopontin</td>
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<td>P4HB</td>
<td>Poly 4-hydroxybutyrate</td>
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<tr>
<td>PAVIC</td>
<td>Porcine aortic valve interstitial cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Personal computer</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
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<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<td>Poly(ethylene glycol)-diacrylate</td>
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<td>PLLA</td>
<td>Poly-L-lactic acid</td>
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<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PUA</td>
<td>Poly(urethane acrylate)</td>
</tr>
<tr>
<td>TEHV</td>
<td>Tissue engineered heart valve</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TMSPMA</td>
<td>3-(trimethoxysilyl)propyl methacrylate</td>
</tr>
<tr>
<td>USB</td>
<td>Universal serial bus</td>
</tr>
<tr>
<td>UTS</td>
<td>Ultimate tensile strength</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
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<td>Vascular cell adhesion protein 1</td>
</tr>
<tr>
<td>VIC</td>
<td>Valve interstitial cells</td>
</tr>
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<td>w/v</td>
<td>Weight per volume</td>
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Chapter 1

1 Introduction

1.1 Background and motivation

Cardiovascular diseases (CVDs) are currently the leading cause of death worldwide and thus represent a major health concern, especially among the elderly population. In 2004, 17.1 million people died from CVDs, accounting for nearly 30% of all deaths globally [1]. It is projected that by the year 2030, more than 23 million people will die from CVDs annually [1]. The most common CVDs are coronary heart disease, cardiomyopathy and aortic valve diseases (AVDs). Of these, AVDs account for ~15,000 yearly deaths in the United States with more than 100,000 surgeries performed locally and 300,000 surgeries globally [2]. With the mean cost of one surgery of $140,000, around $14 billion dollars are spent yearly in the United States to treat AVDs [3]. Calcific aortic valve disease (CAVD), the most common AVD, is a widespread syndrome that currently affects an estimated 25% of the population aged 65 years and up [4]. It is therefore of great interest to invest money and time into ground breaking cardiovascular research in order to tackle this ‘global killer’.

Under normal physiological conditions, the aortic valve cycles ~3 billion times in one lifetime. The valve’s function is to control unidirectional oxygenated blood flow from the left ventricle to the aorta. Its special geometry allows the valve to behave like a mechanical check valve: high pressure in the left ventricle opens the valve during systole, whereas high pressure in the aorta closes the valve during diastole, preventing backflow of blood into the ventricle. In the diseased state, extensive remodeling increases the stiffness and thickness of the valve and this condition is known as sclerosis. In some cases, sclerosis can progress to stenosis, a state that is described by the clinically significant narrowing of the blood flow passage due to severe thickening and stiffening of the valve leaflets. At this stage, the functionality of the valve is severely hindered and the heart must work at hyper-physiological limits in order to push the same volume of blood through a smaller area. This directly leads to increased blood pressure and can ultimately cause cardiac hypertrophy (enlargement of the heart) followed by heart failure and death. Unless promptly treated, the life expectancy of 90% of patients with advanced aortic valve stenosis is at most 10 years, and 50% will die of heart failure within the first year [5].
The current available treatments cannot remedy sclerosis and the only feasible options are to undergo valve transplantation with either mechanical or biological substitutes. Mechanical valves have been well established as a valve substitute that is structurally robust as it does not disintegrate or degrade inside the body. However, the main disadvantage with these valves is the occurrence of thrombogenesis (blood clotting) and patients have to be subjected to a lifelong treatment with anticoagulants. Furthermore, young patients cannot take advantage of this option because they require constructs that will continually grow and adapt at the same rate as the children age. Biological substitutes (homografts or xenografts) do not give rise to thrombogenesis and there is evidence that they can remodel and grow with the surrounding tissue in vitro [6]. Although they appear to be more feasible than mechanical valves, biological substitutes frequently generate immune responses that cause inflammation. An ideal solution would therefore be to harvest cells from the patient and grow a new functional valve that can be ultimately transplanted into the patient. This approach is called tissue engineering. Tissue engineering attempts to restore, maintain and improve tissue function by developing biological substitutes in platforms called bioreactors [7]. Bioreactors are systems that provide stimuli and physiological conditions to growing constructs. Bioreactors are also used to test the functionality of developed constructs or to investigate pathological progression in native tissue. However, few bioreactor systems exist that can replicate the native physiological environment well and provide multiple and integrated stimuli efficiently.

Throughout its life, the aortic heart valve is subjected to a range of mechanical forces (tensile, flexural, etc.) and varying hemodynamic environments (shear stresses and disturbed flow). The maintenance of valve structure and function in this unrelenting, physiologically demanding environment can then only be achieved by a complex set of interacting elements. It has been shown that matrix stiffness, mechanical strain and chemical stimuli can all play a significant role towards regulating the cellular events in the valve fibroblast and can change valve functionality and initiate remodeling of tissue [8-14].

Thus, we have developed a platform that can integrate mechanical straining with different matrix conditions and various chemical stimuli in a high-throughput and combinatorial manner. The platform could be used to engineer tissue but we have focused on combinatorially screening valve cells for the presence of pathological markers in response to the aforementioned integrated factors.
1.2 Objectives

The overall objective of this study was to design a system used to combinatorially expose porcine aortic valve interstitial cells (PAVICs) to *in vivo* like stimuli, and perform experiments investigating their effects on valve pathology. The overall project goals were thus the following:

- Conceptually design and manufacture a bioreactor system for multifactorial stimulation (i.e., regulating tensile straining, matrix stiffness and biochemical stimuli).

- Investigate the effects of strain, biochemical stimuli and matrix stiffness on PAVIC expression of pathological markers.

1.3 Thesis organization

The first chapter presents the background and motivation of the study. The thesis objectives are also outlined here. Chapter 2 is a review focused on the current state of bioreactors pertaining to tissue engineered heart valves (TEHVs) as well as on hydrogel systems commonly used in microscale tissue engineering. Chapter 3 describes the design and setup of the bioreactor, as well as its integration with an actuation mechanism. Chapter 4 focuses on the design, fabrication and strain characterization of the bioreactor substrate. Chapter 5 describes the process of hydrogel selection and its integration with the bioreactor substrate. This chapter also looks at the strain characterization of the hydrogel. Chapter 6 examines the main biological studies that were investigated using high-throughput conditions in the bioreactor system. Chapter 7 outlines the conclusions and future recommendations.
Chapter 2

2 Literature Review

2.1 Introduction

The term bioreactor is generally assigned to mixed closed culture environments, the variables of which can be strictly controlled to direct biological and biochemical processes. In large-scale applications (e.g., fermenters), the biologically active environment involves eukaryotic or prokaryotic cells, and is subjected to various conditions to grow the cells to high densities for producing enzymes or different metabolic products [15]. In the context of industrial biotechnology, specifically for the aforementioned purposes, there are several types of bioreactors such as stirred tank, packed beds and membrane bioreactors. The most common type is the batch stirred tank reactor [16]. Bioreactors are also commonly employed in wastewater treatments, pharmaceutical industry, food processing and the production of recombinant proteins [17].

Tissue engineering has been defined as the application of engineering and life sciences principles and methods for the development of biological substitutes to restore, maintain or improve tissue function [7]. In the context of tissue engineering, bioreactors are used to provide highly reproducible cellular and tissue proliferation. The general goal of tissue engineering is to create fully functional three-dimensional (3D) tissue structures from two-dimensional cell cultures. Relevant cellular biological and biochemical activity is ensured by controlling a series of parameters that can simulate in vivo environments. Strict control in directing cell fate is achieved by varying temperature, pH, gas exchange and concentration, media flow rate, biochemical gradients and mechanical stresses. The final tissue engineered construct should exhibit biologically relevant architecture (size/shape), biochemical activity and composition, biological function and morphology [15]. In the most general approach, 3D tissues are realized through the in vitro culture of porous scaffolds and the rigorous control of the aforementioned parameters. By also applying appropriate biomechanical stresses, cellular activity and phenotype can be directed to generate functional grafts that can replace damaged or lost body parts, giving rise to functional tissue engineering [18]. Tissue engineering of 3D constructs can be also used to create non-implantable devices to provide external organ support whenever donors are not readily
available [19]. It has been shown that tissues continually modify their structure, adapt their shape and change their composition based on the external stimuli that are applied [20]. Therefore, a biologically relevant bioreactor is not one in which one cell type is mass cultured in an immobilized manner, but rather one in which all factors that dictate cellular development are choreographed and integrated to produce the sufficient growth of a multitude of cell types into an organized and interactive 3D structure.

This chapter first reviews general bioreactor design requirements, and then focuses on bioreactors developed for the cyclic straining of aortic heart valve constructs, the focus of this thesis. Dynamic stimulation is crucial during the maturation of tissue engineered heart valves (TEHVs) since there is evidence that mechanical stimuli alter matrix remodeling and cause TEHVs to exhibit structural and biological properties similar to native tissue [21-25]. Finally, the latter part of the review outlines a number of hydrogel systems that have recently been extensively used in combination with bioreactor systems in microscale tissue engineering [26], an approach used in this thesis.

2.2 General requirements and important aspects in bioreactor design

In order to satisfy requirements of reproducibility, scalability, reliability and product safety [17, 27], a typical bioreactor should offer precise control over a number of internal environment-controlling variables. Industrially, bioreactors have been well established for the culturing of cell and microbial colonies, but in the context of tissue engineering, standardized parameters may not be applicable. Three-dimensional tissue engineered constructs possess different requirements based on the type of cell line used (e.g., cartilage, bone, skin) and may even require individual designs [28]. The design of such reactors requires an integrated approach that carefully combines engineering and life sciences concepts. While there is no set of strict rules that dictate the design of bioreactors for each cell type, a set of guidelines aiding the design process is well established. Generally, bioreactors for tissue-engineered constructs should provide an environment that encourages cells to proliferate and differentiate at rates similar to in vivo conditions. In addition, it is critical to maintain a rich and constant supply of nutrients, as well as to ensure that seeded cells are uniformly disseminated in 3D porous scaffolds, which serve as templates for the tissue. An important aspect is also the mechanical conditioning of tissues throughout their maturation
and bioreactors should exhibit this feature whenever applicable [29]. To achieve robust environmental control, a series of parameters should be kept at values close to in vivo conditions. As such, values for internal temperature, media flow rate, pressure, humidity, pH and nutrient and oxygen concentrations need close supervision. Aseptic operation that prohibits the entry of foreign bodies such as bacteria must also be achieved. Ideally, feeding and sampling would be automated processes, although numerous designs employ manual nutrient and media replenishing protocols. The following three basic precautions are generally suggested to maintain aseptic operating conditions: (i) pre-sterilization of equipment, (ii) preparation of sterile media and (iii) maintenance of sterility during culture [28].

A particularly challenging design requirement is the realization of highly efficient mass transfer rates during the growth of the tissue-engineered construct. The absence of vasculature imposes severe constraints on a spatially uniform nutrient supply to the growing tissue. The main mechanism of mass transfer is therefore diffusion [16, 30], and it has been shown that nutrients, specifically oxygen, poorly penetrate tissue at a depth greater than 100-200 µm [31]. As the size of engineered tissues increases, the issue of mass transfer becomes pivotal in establishing a construct with physiological properties.

Depending on the nature of the native biology that the tissue is trying to mimic, bioreactors may involve intricate systems requiring feeding pumps, vessels for media and control strategies. For example, the assembly of pulsed flow bioreactors, which are mainly utilized for growing vascular grafts and cardiac valves, includes all the aforementioned components. It is then important to design a system that can fit in commercially available incubators and that can be easy to operate to maintain aseptic conditions throughout. In addition to the technical requirements imposed on the design of bioreactors, cultivation of cultures for use in clinical trials and subsequent studies should meet good manufacturing practices and quality assurance guidelines [15, 27, 28].

2.3 Tissue Engineered Heart Valves

2.3.1 Goals

Cardiovascular disease is one of the most widespread causes of mortality worldwide and therefore represents a major health care issue. In 2005, according to the World Health
Organization, 17.5 million fatalities occurred due to cardiovascular disease, which represents 30% of deaths worldwide [2]. Among all cardiovascular diseases, diseases of the heart valves are relatively common, with more than 170,000 heart valve replacement procedures performed annually worldwide [32], thereby motivating the development of state-of-the-art valve substitutes. The aim is to create valve constructs that possess the ability to grow, repair and remodel with the patient, do not present immunogenic potential and do not give rise to thrombogenesis. The most challenging part of the problem is finding appropriate valve substitutes for paediatric patients that require tissues that will continually adjust and grow as the patient gets older. At the moment, mechanical, biological and polymeric prostheses are widespread in use but each category presents several disadvantages. While mechanical prostheses are very durable and cannot be affected by calcification or fibrosis, the intricate nature of the designs gives rise to thrombogenesis, requiring the patient to carry a lifelong therapy of anticoagulants. The rigid structure of the valves also disturbs local hemodynamic flows (which could also contribute to thrombogenesis) and interacts non-physiologically with the surrounding native tissue [33]. Evidently, this option is unsuitable for young patients since it cannot adapt to the growing tissue. Biological prostheses can be either homografts (used in the Ross procedure) or xenografts; while they do not have thrombogenic issues, immunogenicity is frequently a problem. In addition, the lifetime of these substitutes is shorter because they are susceptible to calcification. Although this option offers some drawbacks, there is a general agreement that biological substitutes are preferable over mechanical or synthetic ones [33-37]. It has also been shown that biological prostheses have the ability to grow with the surrounding tissues and organs [6]. Tissue engineered heart valves therefore are a variant of the biological substitutes, whereby new valves are grown in vitro in specifically designed bioreactors that stimulate differentiation and proliferation of the patient's progenitor cells (e.g., mesenchymal, bone marrow). Engineering readily implantable heart valves requires, among other things, an understanding of the mechanical forces that act on the native tissue in vivo, to simulate those stimuli in customized bioreactor systems.

2.3.2 The aortic heart valve – anatomy and function

The aortic heart valve is located between the left ventricle and the aorta, and consists of three semilunar cusps that simultaneously open and close during one heartbeat. The valve operates similarly to a mechanical check valve, allowing flow of blood when pressure in the left ventricle
increases, and closing shut when the pressure is lowered (Figure 2-1). In healthy valves, backflow of blood or regurgitation is prevented via valve coaptation, the process by which the valve leaflets seal in the middle at a juncture called the commissure. The base of the leaflets is attached at a fibrous annulus that is connected to the distal end of the left ventricular outflow tract [38].

**Figure 2-1** – The aortic valve consists of three leaflets that open and close at the commissure (A) based on the pressure difference between the left ventricle and the aorta (images adapted from www.nucleusinc.com).

Throughout the average life of a human, the valve opens and closes (at \( \sim 1 \) Hz frequency) around three billion times in an environment that is extremely physiologically demanding [39]. During diastole, which is when the heart relaxes, the transmural pressure (differential pressure between the two sides of the valve) can reach values as high as 100 mmHg [40]. Furthermore, the valve sustains an additional number of mechanical stresses throughout one cycle: flexure during valve opening, laminar and disturbed flow while it is open, flexure during closure, and tension as the
valve coaptates [41]. Evidently, maintaining normal heart valve function and integrity is challenging, and this can only be achieved by a specialized structural composition.

Each valve leaflet consists of three distinct layers: the ventricularis, spongiosa and fibrosa [42], and each of these layers have different mechanical characteristics due to their extracellular matrix composition (Figure 2-2, modified from [43]). The fibrosa layer is on the aortic side of the valve and is made up of circumferentially oriented collagen fibers. These coarse fibers form macroscopic folds that run parallel to the free edge of the leaflet and are the strongest part of the valve, mainly responsible for bearing diastolic stress [44]. The middle layer, spongiosa, is mainly composed of glycosaminoglycans and loosely arranged collagen fibers. The layer closest to the ventricle, the ventricularis, consists of collagen and a higher proportion of elastin fibers that are oriented radially. These fibers give the ventricularis elasticity, which is important to give the valve low flexural rigidity and restore the contracted configuration of the leaflet during systole [38]. The abundance of glycosaminoglycans in the spongiosa makes it suitable for cushioning through shock absorbance and accommodation of relative movement between the outer layers [45, 46].

![Figure 2-2](image)

Figure 2-2 – Cross section of a healthy valve stained with hemotoxylin and eosin depicting the three structural layers (a), endothelial cells and interstitial cells on the aortic side (b), and endothelial cells and interstitial cells on the ventricular side (c) (arrows point to the location of the endothelial cells) (modified from [43]).
At the cellular level, the valve is principally composed of valve interstitial cells (VICs) and endothelial cells (Figure 2-2), although small proportions of smooth muscle cells and nerve cells have been noted [38]. The endothelial cells line the inflow and outflow surfaces of the valve and their roles are to transduce shear stresses, to provide thromboresistance and mediate inflammatory response, and to transmit nutrients and biochemical signals to the valve interstitial cells (VICs) [47]. Quiescent VICs occupy the valve interstitium in heterogeneous populations that have the potential to differentiate to multiple lineages [48]. The homeostasis of the heart valve is largely due to the actions of VICs, which express matrix-degrading enzymes (including matrix metalloproteinases and their inhibitors), secrete growth factors and cytokines, and remodel, repair and maintain the valve tissue during physiological low levels of injury [38, 46, 47, 49]. During normal wound healing, the interstitial fibroblasts differentiate into contractile myofibroblasts that contribute to tissue repair and matrix remodeling [50]. The contractile nature of the myofibroblasts arises from the expression of alpha-smooth muscle actin (α-SMA), which is a protein typically found in smooth muscle cells. Myofibroblast differentiation occurs in the presence of mechanical tension and transforming growth factor-beta 1 (TGF-β1) and their effects are synergistic [9, 51]. In our group, we have also shown that VIC function can be modulated by matrix stiffness and external mechanical loading [8, 12]. These players all interact to dynamically change the microenvironment of the aortic valve and can lead to pathological conditions [52].

Before tissue engineered constructs can be fully realized, a deep understanding and control of the factors controlling tissue maturation as well as pathogenesis must be achieved. To this extent, platforms that can simultaneously grow tissues as well as test their functionality and pathological potential have been developed. The next section highlights some of the latest studies.

2.3.3 Cyclic straining bioreactors

As previously mentioned, mechanical conditioning of valve constructs can be used to establish a structurally and physiologically relevant engineered tissue. Considering the main types of loads present in one heart beat cycle, a number of investigations have been conducted in which the effects of combined and individual stimuli were studied. Studies conducted by Mol et al. [53] and Schmidt et al. [54] made use of a cyclic straining bioreactor meant to mimic the diastolic phase of the heart cycle (Figure 2-3, adapted from [53]). The application of transvalvular
pressure simulated the dynamic pressure exerted on the closed native valve after coaptation. Mol et al. [53] found that both prestrained (via scaffold acting as stent) as well as prestrained + dynamically strained engineered valves exhibited improved tissue morphology and greater collagen synthesis compared to statically cultured leaflet strips. The dynamic strain was achieved via a pressure wave whose shape and frequency were controlled for physiological transvalvular pressures. Ultimate tensile strength (UTS) and modulus (E) for prestrained (0.25 ± 0.02 MPa & 1.03 ± 0.03 MPa) and prestrained + dynamically strained (0.22 ± 0.04 MPa & 0.78 ± 0.2 MPa) leaflets were statistically higher than non-conditioned strips (0.07 ± 0.03 MPa & 0.18 ± 0.07 MPa). This trend was in agreement with the findings by Schmidt et al. [54]. While Mol et al. [53] did not quantify the extracellular matrix (ECM) elements, the dynamically strained leaflets appeared more homogeneous and dense than prestrained or static conditions.

Figure 2-3 – Schematic drawing of the diastolic pulse duplicator bioreactor system used by Mol et al. [53] (a), and a photograph of six pulse duplicators running simultaneously (b). The pulse duplicator consisted of a bioreactor where the constructs were cultured (A), a medium container (B), tubing (C) and a roller pump (D). Part of the upper tubing was connected to a polycarbonate cylinder (E) that was filled with air via a magnet valve (F) and this compressed and decompressed the tubing, creating a pressure difference across the valves. A syringe is used as a compliance chamber (G) and pressures were monitored using pressure sensors (H).
Slightly different results were reported by Schmidt et al. [54], who found that hydroxyproline and DNA contents of strained valves were only 14% and 68%, respectively, of those of native tissue. This discrepant finding could be explained by the variation in transvalvular pressure magnitude. Mol et al. [53] used pressures up to 80 mm Hg while Schmidt et al. [54] kept a constant value of 15 mm Hg. Hence, morphological differences might be influenced by the need to maintain a minimum pressure threshold. A more recent study by Syedain [55] used a comparable conditioning procedure whereby valve constructs were cyclically pressurized with culture medium. The substrates were attached to a stiffer latex tube to achieve pressure-induced stretching. Applied pressure and resulting strain were monitored and incrementally increased throughout the conditioning period to account for increased construct stiffness due to tissue maturation and scaffold degeneration. Valve constructs were conditioned in three steps with incrementally increasing strain amplitude (5%, 10% and 15%) through a sinusoidal wave given by a syringe pump. Values for circumferential UTS and E were 97% and 77% significantly higher for stimulated constructs than static ones. UTS was 50% lower than native tissue but E was not statistically different. However, radial tensile properties and leaflet thickness were not statistically different from either static controls or native tissue. In addition, maximum tension and membrane stiffness were improved. Compared to static controls, they were statistically different but not different from native tissue. The index of anisotropy was also comparable to physiological values. Collagen density was 86% greater than static and only 37% lower than native tissue. The bioreactor developed by Syedain [55] therefore could condition, with constant strain-amplitude, TEHVs that displayed similar properties to ovine pulmonary valves, eliminating the need for complex feedback control systems. Strain-amplitude is an important variable to control since both scaffold degradation and tissue maturation correlate with it [10, 56]. In another study, Mol et al. [57] utilized another cyclic straining bioreactor that conditioned the tissue constructs longitudinally in a custom-built design, with strains of up to 10%. For strains equal or larger than 9%, it was found that the DNA content, hydroxyproline, glycosaminoglycan content, UTS and E were all statistically higher than static controls. These results support the aforementioned evidence that cyclic straining is a relevant, physiological and critical step towards establishing viable TEHVs. Additionally, conditioning with higher magnitude strains may improve tissue formation to a higher extent than with low magnitude strains.
Cyclic flexure represents a major loading mode for the native heart valve, occurring during systole and diastole [38, 46, 58]. Independent flexural effects have been poorly investigated, as most of the research has been performed using pulse duplicator bioreactors, which mimic physiological flow and pressure waveforms. However, the effect of these two parameters is intrinsically coupled and cannot be directly related to other individual loading types (e.g. shear and cyclic flexure). In a first study, Engelmayr et al. [59] developed a bioreactor designed to apply uni-directional or bi-directional three-point flexure (Figure 2-4, modified from [59]). The linear actuator driven bioreactor could stretch up to 12 samples with control over frequency, amplitude, acceleration and deceleration. They studied the effects of flexure on non-woven polyglycolic acid (PGA)/poly-L-lactic acid (PLLA) scaffolds coated with poly 4-hydroxybutyrate (P4HB), which are frequently used biodegradable polymers in TEHVs, and found that degradation of the scaffolds was more pronounced in the dynamic control than in the static one, implying that mechanical loss is accelerated by dynamic conditions. This suggested that these differences need to be taken into account for comparing the effects of cyclic flexure on TEHVs. In a follow-up study, Engelmayr et al. [60] showed that cyclic flexure can

![Figure 2-4](image_url)

**Figure 2-4 – Photograph of the bioreactor system used by Engelmayr et al. [59] depicting the setup consisting of two parallel chambers anchored to a base plate and coupled via a cross bar to a centrally located linear actuator (a), and schematic of a single chamber in the bioreactor (b). The chamber was comprised of six culture wells (C) wherein four orthogonal pins were positioned around a central channel. Through the displacement of the actuator (not shown), which translated its motion to the arm (A), “flexure pins” that could be inserted into the fingers (D) would protrude into the wells to actuate the samples in a given way. The interior of the bioreactor is shown in (B) (modified from [59]).**
independently affect the *in vitro* development of TEHVs. Using the same bioreactor system described above (Figure 2-4), it was found that flexed smooth muscle cell-seeded scaffolds had greater E, 63% greater collagen concentration and generally more homogeneous transmural cell distributions compared to static conditions. In this pioneering study, a high linear correlation was also found between E and the collagen concentration, serving as a useful tool for future cyclic flexure studies whereby the modulus could be predicted simply by measuring the collagen concentration.

Balachandran et al. [61] used the bioreactor design by Engelmayr et al. [59] to cyclically and circumferentially stretch strips of native aortic valves (Figure 2-4). A near physiological loading curve was applied according to Thubrikar et al. [62]. Without causing any gross damage to the native tissue, the dynamically stretched valve strips displayed a 15% increase in collagen content and an increase in α-SMA expression in the ventricularis side, compared to fresh and static control leaflets. The increase in collagen percentage was associated with a decrease in the degradation of collagen since the strips were circumferentially stretched, and because collagen fibers are aligned parallel to the circumferential direction and serve as the major load bearer. It was also hypothesized that cyclic stretch might be a regulator of the myofibroblast phenotype, with the ventricularis side containing hot-spots that generate a focal response to mechanical forces. This study recapitulates the idea that isolated effects, such as circumferential stretch, alter the ECM composition of native tissue, causing valve remodeling.

Little is known about the biosynthetic and biochemical activity of the valve tissue during pathological conditions. To that extent, high throughput bioreactor platforms can be used to not only engineer new tissue, but also investigate pathological conditions that could lead to disease. Comparisons between gene and protein expressions for physiological and pathological conditions can further elucidate the mechanisms behind disease progression.

During normal conditions the valve is subjected to 10% in the circumferential direction [38] strain during diastole and preliminary studies confirmed an increase of 5% in strain for every 40 mmHg rise [13]. Several studies have reported that cyclic stretch influences collagen production at the cellular level. Merryman et al. [63] found that VICs respond to local stresses by altering the valve stiffness via increasing the synthesis of collagen. These results are in agreement with Ku et al. [64] who demonstrated that cyclic stretch upregulates collagen synthesis in VICs.
Merryman et al. [107] also found that the presence of certain cytokines such TGF-β1 causes valve matrix remodeling that is associated with a compromise in valve function and could signal disease initiation. Matrix metalloproteinases and their inhibitors (proteolytic enzymes), as well as cathepsins, are overexpressed in diseased aortic cusps [65]. These proteins are generally involved in cellular processes such as proliferation, apoptosis, cell differentiation and have been shown to play a role in disease progression [66]. A recent study by Balachandran et al. [13] compared strips of circumferentially cut valve tissue subjected to 5 conditions: fresh, static, 10% stretch (physiological), 15% stretch (pathological/hypertensive) and 20% stretch (hyperpathological/severely hypertensive). It was found that cathepsin L was downregulated for elevated cyclic stretch (15% and 20%) whereas cathepsins S and K were upregulated. An increase to pathological levels in cell proliferation and apoptosis was also observed at the elevated cyclic stretch conditions. In addition, elevated stretch conditions also exhibited a significantly higher level of matrix metalloproteinases (MMPs) but lower level of tissue inhibitors of metalloproteinases (TIMPs). Physiological levels of stretch maintained normal expression of matrix remodeling proteins, in contrast with an increase of collagen synthesis for the elevated stretch conditions, in agreement with Ku et al. [64] and Merryman et al.[63]. However, the collagen increase for the specified levels of the strain correlates with Ku et al.’s [64] results very well, who measured levels of [3H]-proline incorporation to quantify collagen expression. In a further study, the same group [14] investigated the effect cyclic strain and that of serotonin/5-hydroxytryptamine (5-HT), which has been implicated in valve disease and modulation of valve mechanical properties. Valve cusps were cyclically stretched at 10% and 15% with and without the presence of 5-HT. It was found that collagen synthesis, cell proliferation, and tissue stiffness all increased upon the application of cyclic stretch and 5-HT. Several studies were also performed on the expression of vascular cell adhesion-1 (VCAM-1) mRNA expression and osteopontin (OPN) mRNA expression in cyclically loaded VICs subjected to hypertensive conditions [67-71]. It was found that VCAM-1, responsible for adhesion, migration and accumulation of monocytes and T-cells, had an increase in mRNA expression relative to static controls, whereas OPN, which inhibits mineralization, exhibited a decrease in its mRNA expression. This could suggest a pathological reaction, associated with increased mRNA expression of VCAM-1 and that OPN might not be involved in ectopic calcification. Therefore, these studies underscore the ability of the aortic heart valve to modify its matrix components to function appropriately in a varied mechanical environment, as well as the
important response of the VICs to regulate protein expression; hence, these data could potentially correlate with disease-modeling pathways.

2.3.4 Summary

Heart valve constructs exhibiting mechanical and structural properties that closely resemble the properties of native tissues have been developed by applying stimuli such as straining due to transmural pressures, flexural straining, circumferential straining, tensile straining or a combination thereof. These mechanical stimuli were also used to investigate the initiation and progression of pathological conditions on strips of native tissue. However, mechanical conditioning of valvular constructs or native tissue causes responses at the cellular level that are influenced by other microenvironmental stimuli and therefore are best studied by considering their integrated influence on cell and tissue fate and function. Current heart valve bioreactor systems cannot be used to efficiently study the effects of multiple mechanobiological stimuli on cell and tissue responses. This knowledge and technology gap is addressed in this thesis by developing a high-throughput platform that is able to apply not only mechanical loading but also can subject substrates or cells to different matrix and biochemical conditions. The advantages of such systems are twofold: 1) stimuli working in concert may better represent the \textit{in vivo} complex physiological environment; and 2) gathering data from multiple samples in one experimental run minimizes experiment-to-experiment variability and can significantly reduce the number of experiments performed.

2.4 Microfabricated hydrogels for tissue engineering applications

In conventional tissue engineering, the main approach is to recreate the physiological environment by seeding cells onto biodegradable scaffolds that resemble the native ECM [7, 22, 24, 72, 73]. Hydrogels have been studied extensively within tissue engineering due to their excellent properties in mimicking the physical, mechanical and biological properties of native ECM [26, 74-76]. Advances in microfabrication and microfluidics have encouraged a merger between microfabrication techniques and biomaterials to create a powerful approach toward reproducing the physiological environment [77]. Microfluidic hydrogels created via microneedle template, fiber template, soft lithography, bioprinting, and photopatterning have been reported [78]. Natural biomaterials (e.g., collagen, agarose, alginate, chitosan, gelatin and fibrin) as well as synthetic ones (e.g., poly(glycerol sebacate), poly(D,L-lactic-co-glycolic acid), poly(ethylene
glycol), poly(acrylic acid), etc.) have been used [79-81]. This section reviews commonly used hydrogel systems and outlines the employed microfabrication/micropatterning techniques and possible applications to tissue engineering.

### 2.4.1 Collagen

The physiological origin of collagen, combined with its low antigenicity, biodegradability, abundance of cell-binding domains, significant compaction that yields high cell density, and mechanical compliance have contributed to its widespread use as a matrix scaffold in macro- and microscale tissue engineering [82, 83].

Conventional approaches employing soft lithographical techniques [83-85], microfluidics [86-88] or the combination thereof [89] have been used to create cell-laden collagen gels. Legant et al. [84] first described a microfabricated device based on rectangular polydimethylsiloxane (PDMS) wells in which microcantilevers were soft lithographically patterned. The group seeded 3T3 fibroblasts mixed with collagen solution and a self-assembled tissue was formed due to gel compaction. By independently varying the stiffness of the cantilevers and the collagen matrix, they found that forces exerted by the cells increased with tissue matrix and boundary rigidity but cytoskeletal and ECM proteins correlated with mechanical stress. A more recent study on the same platform [85] also investigated the effect of electrical stimulation and auxotonic loads on cardiac microtissues formed from rat neonatal cardiomyocytes. They showed that the aforementioned factors strongly improved the functionality and structure of the microtissues, closely emulating the native myocardium. Three-dimensional cell-laden microtissues such as the ones described are essential in drug-delivery studies with low-volume requirements. The same group [85] went on to demonstrate this utility by monitoring drug-induced changes in spontaneous frequency and contractility and employed high speed imaging to track calcium dynamics. Mesoscale cardiac and skeletal muscle tissues were also developed utilizing microfabrication techniques. Bian et al. [83] used a bed of soft lithographically patterned and strategically placed 2 mm pillars to engineer a tension-mediated aligned tissue. Therefore, such larger scale approaches that still employ microfabrication techniques to achieve physiological properties can also be important in organ repair applications.

In a microfluidic approach, Huang et al. [86] developed a microdevice containing a micropillar array that allowed the formation of apposed collagen I and Matrigel™ hydrogels. Metastatic
cancer cells and RAW264.7 macrophage cells were encapsulated in the hydrogels to study the cell-cell and cell-matrix interactions within the array of alternating gels. It was found that the macrophages invaded the gels containing cancer cells but not the gels lacking cells. The hydrophobicity and surface wettability of the PDMS microchannel were monitored to prevent leaking of hydrogels to a neighboring location thereby achieving spatial control and compartmentalization. Migration of tumor cells in response to interstitial flows has also been investigated on a microfluidic chip [87]. Breast cancer cells were cultured in collagen I and the cell-laden hydrogel was subjected to interstitial flow. Random migration of the cells was reported without flow whereas migration followed the flow streamlines when flow was applied. However, when the CCR7 receptor was blocked, the cells migrated against the flow, demonstrating a CCR7 dependent migration with interstitial flow. Microfluidic approaches thus enable the fabrication of cell-laden hydrogels and allow for the regulation of geometrical, physical and chemical parameters.

Platforms containing microneedle template fabricated collagen microchannels have been used to study the effect of cyclic adenosine monophosphate (cAMP), which controls the barrier function of microvessels, on endothelialized conduits [90, 91]. Wong et al. [91] conducted experiments testing the permeability and local leaking of microvessels to fluorescently-labeled bovine serum albumin (BSA) solution. It was found that cAMP decreased the permeability and local leaking but enhanced mechanical stability of the vessels. Also, cAMP inside the conduits suppressed cell division and apoptosis. Therefore, vascular function and phenotypes can be regulated using collagen derived conduits. To overcome the mechanical softness of collagen at time points in the early remodeling process, collagen-containing poloxamine hydrogels that could be chemically crosslinked were developed as a proof of concept [82]. Human hematoma HepG2 and endothelial cells adhered to, spread on the surface and maintained high viability levels after 24 hours of culture.

Collagen is therefore a primary choice in microscale tissue engineering mainly due to its biocompatibility, biodegradability, abundance of cell-adhesion ligands, and ability to be remodelled by residing cells into self-assembled, functional tissues. However, its ability to maintain long-lasting micropatterns that are subjected to external stimuli (e.g., mechanical straining) is typically limited to its insufficient mechanical integrity.
2.4.2 Alginate

Alginate is a polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid monomers [92] and is naturally found in the cell walls of brown algae. Its ability to absorb large quantities of water and form gels from aqueous solutions when in contact with divalent cations, such as calcium ions, have encouraged its use as a possible tissue engineering material. Studies have also shown that gelated forms of alginate, most commonly calcium alginates, support long-term cell culture and formation of functional tissue, exhibit moulding capabilities to form complex cell-laden structures and can be chemically functionalized with cell-binding ligands [93], making it an attractive candidate as a scaffolding material for tissue engineering.

The creation of alginate microfibers using microfluidic platforms has been reported [94-96]. For example Shin et al. [95] used a microfluidic chip containing a capillary glass pipette to create co-axial flow based microfibers. The fibers were created using a sample flow of 2% (w/v) sodium alginate solution and a sheath flow of a 100 mM CaCl2 solution. The diameter of the fibers could be controlled by adjusting the flow rates of the fluids. To demonstrate utility with respect to tissue engineering, human fibroblasts were encapsulated and exhibited >80% viability after 24 hours. Furthermore, hollow fibers were created by Lee et al. [94] through the introduction of an additional fluid during the fiber formation process. The co-axial microfluidic device consisted of a core flow of a 100 mM CaCl2 solution, sheath flow of 2% alginate solution, and a sample flow of another 100 mM CaCl2 solution. Human iliac vein endothelial cells were embedded into the fibers and then co-cultured with human iliac vein smooth muscle cells in an agar-gelatin-fibronectin matrix. Creating hollow microfibers can have tremendous potential towards tissue engineering microvessels and studying angiogenesis in artificial tissues. Moreover, microfiber architecture is more physiologically relevant as it resembles the structure of collagen. A slightly different approach was reported by Kang et al. [96] who soft lithographically patterned microfluidic channels and co-axially produced alginate fibers in a parallel manner. By eliminating the need of glass capillaries, the production of which is labor intensive, they simplified the process and provided a useful way to scale up the fabrication.

Calcium alginate has also been used to fabricate 3D microfluidic scaffolds via soft lithography [93] and microfluidic chips [92, 97-99]. Choi et al. [93] incorporated soft lithographical moulding techniques to generate a cell-laden 4% calcium alginate 3D scaffold. The scaffold
contained 100 µm wide microchannels that were flushed with either reactive or non-reactive solutes to characterize the convective and diffusive mass transfer of small and large solutes within it. They found that spatial and temporal control of the convective mass transfer was key towards modulating the metabolic environment. Systems exhibiting tight spatial and temporal control of concentration gradients are critical in drug delivery studies. Manipulating the shape of the scaffolding is important in finding efficient methods for perfusion and nutrient delivery to encapsulated cells. To this extent, Wang et al. [97] used a microfluidic method to fabricate a honeycomb-like scaffold in which porcine chondrocytes were encapsulate. The cells aggregated and were viable after 14 days of culture and expressed physiologically relevant levels of ECM. Tight spatial control of scaffolding architecture was further achieved with layers exhibiting resolutions similar to the thickness of a single cell [98, 99]. In an alternative microfluidic approach, Chueh et al. [92] was able to photopattern custom areas of alginate in a microchannel via ultraviolet (UV) light. The process was based on the release of caged calcium from DM-nitrophen upon illumination with UV. Gels with various mechanical integrities were formed by varying the calcium concentration, chelating agents, and the duration of UV exposure. Co-culture of MC3T3 and human umbilical vein endothelial cells was demonstrated. While this approach may be useful in combining photopatterning and microfluidic techniques in alginate hydrogels, the high cost of DM-nitrophen currently limits extensive fabrication of such systems. Non-conventional approaches to microfabricate 3D cell-laden alginate scaffolds through freeform bioprinting using syringe-based systems [100] and inkjet nozzles [101] have also been demonstrated. Simple benchtop fabrication using alginate dipped nylon-meshes eliminated the need for lithography, microarray printing and represented an inexpensive method using off-the-shelf components [102]. Fibroblasts and Schwann cells were grown in 70 µm and 100 µm square compartments, respectively.

In another approach, Tang et al. [103] used microtransfer moulding to create hexagonal features in collagen gels using Matrigel™ as a sacrificial layer, and encapsulated bovine pulmonary artery endothelial cells to demonstrate potential for tissue engineering applications. Another group used a homemade bioprinter to simultaneously deposit collagen and gelatin features simultaneously, layer-by-layer, thereby creating an intricate 3D network of microchannels [104]. These non-conventional approaches can have significant implications in simplifying the methodology for realizing monolithic structures with stringent geometrical requirements.
Alginate is therefore a solid candidate to use in microfabricated and microfluidic systems for microscale tissue engineering due to its ability form highly intricate scaffolds and the presence of natural cell-binding components.

### 2.4.3 Gelatin and gelatin-based hydrogels

Gelatin is a natural by-product resulting from the partial breakdown of collagen that still retains many of the bioactive properties of collagen such as cell-binding domains and, as expected, is biocompatible and not immunogenic. The disadvantage in using gelatin as a scaffolding material to fabricate microstructures or microtissues is that it has weak mechanical integrity at physiological temperatures (37 °C); this is because the gelation temperature of gelatin is below that required for cell culture [105]. Nevertheless, some groups have taken advantage of this property and have used gelatin as a sacrificial material to produce microfluidic hydrogel network platforms [104, 106]. Golden et al. [106] soft lithographically fabricated gelatin meshes with features as fine as 6 μm. Briefly, gelatin was introduced into Pluronic (propylene oxide-ethylene oxide co-polymer) treated and oxidized PDMS channels and gelled at 4 °C. The PDMS mould was then removed and the hardened gelatin was encapsulated in collagen, fibrinogel or Matrigel™. The construct was heated to physiological temperatures causing the gelatin to melt away and leave behind a mesh of interconnected channels. The group also demonstrated enhanced diffusivity of rhodamine and BSA into the channels compared to bulk gels. In addition, the platform supported the adhesion, growth and proliferation of human dermal microvascular endothelial cells. A similar use of gelatin as a sacrificial layer was reported by Lee et al. [104] who used a homemade bio-printer to simultaneously deposit gelatin and collagen patterns layer-by-layer, thereby creating 3D structures. Gelatin was removed upon heating to physiological temperatures and left behind a multi-layered microfluidic construct. Primary dermal human fibroblasts were also cultured for one week and showed increased viability levels compared to scaffolds with no channels. As an alternative to soft lithography, this study offered a more rapid and non-conventional approach to creating interconnected microfluidic channels in hydrogels that has the potential to generate perfusable artificial tissue composites.

Gelatin-derived hydrogels that are stable at room temperature have first been reported by Van Den Bulcke et al. [107] who created a methacrylated version of gelatin, called gelatin methacrylate (gelMA), that was photopolymerizable. The mechanical and rheological properties
of gelMA could be controlled by varying the photoinitiator concentration, degree of
methacrylation or UV irradiation conditions. To this extent, Nichol et al. [108] recently used
gelMA and varied its properties to create cell-laden microarrays and endothelialized tubes,
demonstrating the hydrogel’s potential to generate complex microtissues and co-cultured,
microvascularized tissue constructs. The group achieved a range of stiffnesses (~2.5 kPa to ~30
kPa) that could be controlled by changing the degree of methacrylation and the weight percent of
the prepolymer. Albeit in the high degree of methacrylation the amine groups were close to being
saturated with methyl groups, it is hypothesized that the stiffness of the gels could further be
increased by increasing the gel percentage. It was shown that at ~80% methacrylation and 15
wt% of the prepolymer, human umbilical vein endothelial cells exhibited increased confluency
and density compared to 10 wt% or 5 wt% gels. In addition, NIH 3T3 fibroblasts were
encapsulated in 5, 10 and 15 wt% gels formed by pouring prepolymer between two glass cover
slips using a 150 μm spacer and photopolymerizing using a mask. The cells maintained excellent
viability after two days of culture and showed elongation and spreading. Finally, they created
300 μm diameter perfusable channels using syringe needles. The channels were lined with
human umbilical vein endothelial cells while the surrounding bulk contained encapsulated NIH
3T3 fibroblasts, demonstrating co-culture conditions. In a different study [109], the same group
micropatterned gelMA using a photomask to create rectangular microconstructs of varying
widths. After five days of culture, encapsulated NIH 3T3 fibroblasts aligned in the direction of
the long axis of the construct, exhibiting ~60% nucleus alignment at a width of 50 μm. The
reported required mechanism for alignment was ECM remodeling via an increase of matrix
metalloproteinase-2 (MMP-2) and MMP-9 expression. More recently, poly(ethylene glycol)-
gelMA (PEG-gelMA) constructs were developed [110] as an alternative to the inert and cell-
repellant PEG hydrogel. Adding gelMA to PEG in specific proportions increased spreading and
binding of fibroblasts, favored encapsulation viability, and displayed tunable stiffness and
degradation profiles.

Therefore, gelatin is a great selection of sacrificial material for applications requiring the
fabrication of intricate networks of channels. Correspondingly, the more stable form of gelatin,
gelMA, is a suitable scaffold material in microtissue engineering due to its fine control over the
mechanical properties, the ability to support two and three-dimensional cultures, excellent
micropatterning properties, and natural cell-binding ligands.
2.4.4 Poly(D,L-lactic-co-glycolic acid) (PLGA)

PLGA is a synthetic scaffolding material largely used due to its properties of being rapidly degradable [111] and leaving behind non-toxic agents, making it an attractive hydrogel for microscale approaches to tissue engineering.

PLGA scaffold fabrication techniques have been demonstrated previously by Vozzi et al. [112] with an emphasis on soft lithographical moulding on PDMS. The group developed micro-moulding, microfluidic and spin coating methods for fabrication. The micro-moulding method relied on moulding PLGA solution on a PDMS mould. This yielded the same resolution in both the lateral and vertical directions. In the microfluidic moulding, the PDMS mould was inverted, sealed to surface, and PLGA solution was perfused via negative pressure. In contrast to the previous method, this yielded the same lateral resolution but decreased vertical resolution. The spin-coating method consisted of spin coating PLGA solution on the PDMS mould, and this yielded a significant increase in the lateral resolution but the same vertical resolution as the microfluidic method. Another approach utilizing a pressure assisted microsyringe deposition yielded poor resolutions in both directions. The study also demonstrated the formation of pores via particulate leaching, and therefore an integration of this technique with microfabrication approaches. A similar soft lithographical approach was employed by Yang et al. [113] to create a honeycomb structure designed for drug delivery. To test the functionality of the device, sugar powder was packaged in the micro-chambers of the honeycomb microstructure and it was shown that the scaffold degradation and sugar release rates were linear. A complementary approach was employed by King et al. [114] who fabricated a network of microcapillaries by combining melt micromoulding, to generate thin PLGA films, with fusion bonding, to create monolithic structures.

Studies combining microfluidic and micromoulding approaches of PLGA with cellular studies have also been performed. For example, co-axial flow in a microfluidic device was used to generate PLGA microfibers and control the cellular orientation of mouse fibroblast cells [115]. It was found that the orientation of the fibroblasts, as defined by the angle between the axis of cells and the long axis of the fiber, could be controlled by varying the fiber diameter. Mean cellular orientation angles were reported to increase with increases in fiber diameter. Being able to control the parameters of fibrous scaffolds could be useful in creating tissues where cellular
orientation plays a functional role. Furthermore, McUsic et al. [116] showed that also controlling topographical features on biodegradable scaffolds can drive cellular morphogenesis and differentiation. PLGA scaffolds with features 40-50 µm thick, and 15 µm in diameter, were fabricated by soft lithographically moulding PLGA-acetone solutions of various concentrations in PDMS moulds. Following solvent evaporation, the PLGA scaffolds were removed from their moulds. Postnatal mouse and human embryonic stem-cell derived retinal cells were seeded and confined in the spaces between the scaffold microposts to replicate in vivo like conditions and achieve a basic level of retinal organization. The group assessed the level of differentiation by measuring and confirming the presence of rod, bipolar and Müller glia marker expression and inner/outer cell lamination.

Stackable PLGA microscaffolds enriched with Collagen Type I or Matrigel™ have been demonstrated by Pirlo et al. [117] in a study that evaluated the ability of vascular cells to migrate and infiltrate onto biopaper substrates. First, 300 µm thick PLGA layers were soft lithographically moulded onto a PDMS mould; human umbilical vein endothelial cells were then printed with biological laser printing (BioLP™) technology in intersecting patterns on the surface of the scaffolds. Printed cells exhibited >95% viability after 24 hours of culture on both coated and non-coated scaffolds. After stacking, layers showed significant inseparability suggesting the merging of layers, and the cells occupied a single continuous vertical layer suggesting infiltration and the feasibility of generating layer-by-layer constructs using such constructs. The extensive manipulation of PLGA scaffolds using microfabrication techniques and its degradable and biocompatible properties thus make it an attractive choice for microscale tissue engineering.

2.4.5 Poly(glycerol sebacate) (PGS)

PGS is a tough and biodegradable elastomer that is inexpensive, biocompatible and easy to synthesize [118]. Compared to PLGA, which is mechanically rigid, PGS exhibits improved degradation properties by maintaining its integrity through surface erosion [79]. In studies done by Freed and colleagues [119, 120], 3D accordion-like PGS scaffolds were created by laser microablation. The stiffness of the 3D scaffolds could be precisely controlled by varying the curing time of PGS. In addition, the scaffolds exhibited mechanical anisotropy, which is similar to the native myocardium, that was preserved even after weeks of cyclic fatigue testing. The
group demonstrated the utility of the scaffolds by seeding rat neonatal heart cells that exhibited greater heart cell alignment than isotropic control scaffolds. Heart cell contractility induced by electric field stimulation was also demonstrated within the scaffolds [120]. Additionally, layered constructs were fabricated by adding a second layer via stacking and lamination [119]. Accordion-like scaffolds (both single and multi-layered) thus promoted formation of grafts with aligned heart cells and mechanical properties that emulated the native myocardium.

PGS has also been used to study cellular orientation and alignment of bovine aortic endothelial cells cultured on ridge-groove geometries [118]. The substrates were fabricated by deep reactive ion etching a silicon wafer, coating it with a sacrificial sucrose layer and soft lithographically moulding the PGS on the surface. It was found that the cells cultured on ridged substrates were more elongated and aligned compared to cells grown on flat surfaces, which were randomly oriented. The potential use of this study would be in developing implantable microdevices that further investigate the effect of topographical cues to study cellular alignments. Fidkowski et al. [121] also demonstrated the ability of PGS to be patterned into microfabricated capillary networks. The devices consisted of a patterned PGS layer fabricated in the same manner as described in [118] that was bonded and sealed with a bottom unpatterned PGS layer. The unpatterned layer was attached to glass for rigidity. The devices was seeded and cultured under flow conditions with human umbilical vein endothelial cells and reached confluence within 14 days of culture. Another example of a multilayered microvasculature emulating microdevice is from Bettinger et al. [122] who used PGS as a scaffold to culture hepatocytes. Soft lithographically patterned PGS microchannels were seeded with hepatocyte carcinoma cells and aggregates were shortly formed, thereby increasing opportunity for adhesion of cells to the channels. The function of hepatocytes cultured with perfused medium was evaluated by recording albumin production levels that were similar to physiological values.

Therefore, PGS offers excellent versatility in manipulating its microarchitecture to match the requirements of microfluidic and soft lithography techniques and is a suitable hydrogel for tissue engineering approaches.
2.4.6 Poly(ethylene glycol) (PEG) and PEG-based hydrogels

PEG is a synthetic, photopolymerizable and inert hydrogel that is biocompatible to cells but does not readily support cell adhesion [123] without functionalizing its surface chemistry with cell-adhesion ligands [124-127].

By combining soft lithographical techniques with the cell-repelling surface properties of PEG hydrogels, Karp et al. [123] demonstrated the aggregation of embryonic stem cells into embryoids with controlled sizes and shapes. The template used to generate the embryoids was fabricated by inverting a PDMS mould containing protruding features and pressing it onto a flat and thick PEG polymer layer, followed by UV crosslinking and removal of mould. Embryonic stem cells were seeded at a high density into the resulting microwell arrays (ranging from 40 μm to 150 μm in diameter) and homogenous embryoid bodies formed, remained viable (>95%), maintained their shape and size defined by the geometry of the microwells (10 days), and could be easily retrieved. In a later study, Hwang et al. [128] used a similar platform to show that embryonic stem cell fate could be directed by controlling the size of the embryoid body. Gene and protein expression analysis showed that embryoid bodies in microarrays 150 μm in diameter showed a higher expression of WNT5a (a member of the noncanonical WNT pathway) and this enhanced endothelial cell differentiation. Contrastingly, embroyid bodies in larger microwells (450 μm in diameter) exhibited an increase in WNT11 expression and this enhanced cardiogenesis. Achieving tight control over micropatterning architectures in hydrogels can therefore be a powerful tool to study size-dependent responses of embryoid bodies in a controlled manner.

An alternative to PDMS for soft lithographically patterning nanoscale features is UV curable poly(urethane acrylate) (PUA). PDMS is an excellent patterning polymer for features on the microscale but is unsuitable for features on the nanoscale because it tends to deform, buckle or collapse the relief features due to its low elastic modulus (~1.8 MPa) [79]. Kim et al. [129] used PUA to mould nanoridges with grooves ranging from 150 to 800 nm on a PEG-diacrylate (PEGDA) substrate. The PUA was fabricated by UV-assisted nanomoulding it on a silicone master and using the resulting structure to imprint a sheet of PEGDA to create the nanoridges. The structure of the nanoridges resembled the native ECM structure in the myocardim. After functionalizing the surface with fibronectin, cardiomyocytes were cultured on the patterned
hydrogel and aligned along the ridges. Focal adhesions followed the direction of the ridges and caused the cells to express cytoskeletal proteins that aligned them in the same direction and elongated their shapes. Guided by the topographical cues, the cells exhibited anisotropic action potentials and contractility characteristic of the native microenvironment. Tight control over the cell-substrate contact can advance regenerative medicine to areas that require anisotropic tissue engineering.

Creating patterned structures within PEG hydrogels is not restricted to only using soft lithographical techniques. An important advantage of photopolymerizable hydrogels is the ability to UV cure selected regions of the prepolymer solution, thereby achieving tight spatial control. Studies have been conducted where areas of PEG hydrogels were selectively photocured into various cell-laden shapes using photomasks [130, 131] or field diaphragms and objectives controlling the illumination area [132, 133]. In addition, manipulating the substrate stiffness can have important implications for development, differentiation and disease progression [134]. Accordingly, Cheung et al. [126] used step-wise polymerization to create PEGDA/PEG-fibrinogen substrates with discrete stiffness regions ranging from 0.7 kPa to 50 kPa to investigate human foreskin fibroblast migration. They reported that cell area increased with higher stiffness and that cells migrated towards areas of higher stiffness.

Microfluidic techniques for surface functionalization of PEG hydrogels with biomolecules have also been demonstrated [124, 125]. Specifically, NeutrAvidin and/or Protein A conjugated PEG hydrogels were submitted to hydrodynamic flow focusing in a PDMS microfluidic chip [124]. Varying gradients consisting of biotinylated or Fc-tagged proteins were tethered to the surface, depending on the flow rates of the protein and adjoining buffer streams. After patterning, the flow focusing microfluidic chip was removed and the hydrogels were used for cell culture. Spatiotemporal convective mass transport of nutrients was also characterized in a multilayer microfluidic channel consisting of a soft lithographically fabricated PEG hydrogel [135]. At early timepoints (0 hours and 24 hours), cell viability of NIH 3T3 fibroblasts was high across the width of the channel. However, at 48 hours and 72 hours, only fibroblasts growing closer to the central part of the channel were alive, with peripheral regions (farther than 600 μm from the central region) having undergone necrosis.
Therefore, PEG based hydrogels offer excellent versatility in micropatterning both the micro and nano scales, allowing for both soft lithographical and photopolymerizable patterns. Microfluidic techniques have also been used to either modify the surface chemistry or study mass transport properties of the hydrogel. Having the ability to manipulate the architecture extensively is a tremendous advantage in studying and creating regenerative tissues that demand stringent geometrical and mechanical requirements.

2.4.7 Other types of hydrogels

While the outlined hydrogel systems have been commonly used in micropatterning/microfabrication approaches to microscale tissue engineering, they are by no means the only ones. Important and relevant studies have also been conducted using chitosan [136], fibrin [137], agarose [138], and acrylamide [139] gels. Progress in this rapid changing field has increased tremendously in the last decade and these systems represent the state-of-the-art biomaterials used for engineering and repairing damaged tissues and organs.
Chapter 3

3 Bioreactor Design and Characterization

3.1 Introduction

The overarching aim of this thesis was to develop a robust system with which cells and biomaterials could be subjected to a range of uniform tensile strains, with throughput that enabled combinatorial investigations of mechanical and non-mechanical stimuli. To do so, a tensile loading bioreactor system incorporating microfabricated substrates was designed. The substrates were patterned with cell-seeded hydrogels and then subjected to tensile loading in the bioreactor. As described in the next sections and next chapter, the design of the substrate and bioreactor enabled a range of mechanical, biomaterial, and biochemical stimuli to be investigated combinatorially for their integrated effects on cell response.

3.2 Problem definition and design requirements

Current bioreactor systems, although numerous, are still in their early stages of development. Since the microenvironment surrounding the aortic heart valve is complex and heart valve biology is regulated by multiple factors, this dynamic system is challenging to emulate in vitro. As a result, bioreactors must incorporate a multitude of elements to achieve an efficient integration of simultaneous physiological stimuli. The objective of this thesis project was thus to design a versatile bioreactor that could apply a range of mechanical strains for a fixed displacement (with the option of modifying the loading pattern, if necessary), accommodate substrates of varying geometries and material properties (i.e., substrate stiffness), as well as subject the substrates to various biochemical conditions. The bioreactor would also be able to house a larger number of substrates, if necessary. A further discussion on those parameters is given in Chapter 4, which focuses on substrate design.

The starting point of the design process was a previously in-house developed bioreactor that aimed to stretch macroscopic polyurethane membranes (not published). The main goals of the redesign were to increase the substrate sample size and allow for multiple substrate geometries. Increasing the sample size, as well as being able to tailor the geometry desired substrate, directly
contributed towards the combinatorial nature of the final design. The final design requirements are listed below. In summary, the bioreactor was required to:

- Contain a large number of subcompartments (at least six) to house substrates for combinatorial experiments.
- Be easily connected and interfaced with an actuation device that transmitted linear motion and that could be programmed to achieve physiological frequencies (~1 Hz) as well as amplitudes that stretched the substrates to the required length.
- Accommodate substrates of various geometries.
- Be manufactured from components that can withstand autoclave temperature and pressure (120 °C at 15 psi), as well as resist corrosion in an incubator environment (37 °C, 5% CO₂ and 100% humidity).
- Be manufactured from biocompatible materials that do not leech ions or other elements into the nutrient solution.
- Maintain aseptic conditions during cell culture.
- Provide adequate nutrient delivery to substrates.
- Provide gas exchange between incubator and bioreactor environment without compromising sterility, therefore maintaining pH levels of nutrients appropriate to cell culture conditions.
- Have dimensions that do not exceed those of a standard laboratory incubator.
- Weigh less than the maximum weight an incubator shelf can support.

3.3 Conceptual design

Based on the final design requirements, virtual models of the bioreactor were created in SolidWorks 2010 (Dassault Systèmes SolidWorks Corp.; Waltham, MA, USA). The conceptual model was subjected to iterative design, thereby incrementally altering and improving various aspects to meet all of the requirements. A few conceptual designs with brief descriptions are presented in the following subsections.

3.3.1 Concept 1

The first concept was based on the idea that the nutrient holding containers inside the bioreactor should be disposable six-well plates (Figure 3-1). This would increase ease of use, and would not
require custom-made well plates to be machined and autoclaved before every experiment. The black transversal bars were all welded to the main driving arm. The grey transversal bars were static and they were bolted down to the base. The slots on every transversal bar identified the location of the clamps (not pictured). A clamp attached to the fixed bar would hold one end of the substrate (not pictured), and a second clamp on the moving bar would hold the other end. Therefore, the substrate would be stretched longitudinally. This concept was abandoned primarily because there was not enough space to fit and actuate the substrate within the enclosed space of a six-well plate well.

![SolidWorks model of Concept 1 in isometric view (a), and top view (b).](image)

**Figure 3-1 – SolidWorks model of Concept 1 in isometric view (a), and top view (b).**

### 3.3.2 Concept 2

The second conceptual design was similar to the first one except that the six-well plates were replaced by custom designed well plates (Figure 3-2). Furthermore, the sample number was reduced from 24 to 16 in order to accommodate for larger substrates as well as their displacement during stretch. Linear U-shaped guides were added to prevent non-axial movement of the actuating arm.

The functionality of Concept 2 remained identical to that of Concept 1 with a few minor modifications, most of which were aforementioned. The slots on the transversal arms were extended to run across the well plate for easier installation. It is also noticeable that the clamps had slots machined where they attached to the transversal bars. This was done to increase flexibility in handling the substrate during clamping.
3.3.3 Other concepts

A few other conceptual designs were modeled but they did not contain significant improvements over Concepts 1 and 2, which represented milestones during the iterative design process. The preliminary concepts did not have all the details finalized (e.g., location of screws and exact alignment of components relative to the base) and most of the refinements were performed on higher level features (i.e., maximum number of substrates, clamping mechanism and well plate design). Only when the higher level features were fully determined did the finalization of the concept take place.

3.3.4 Final concept

The main changes in the final concept represented a complete redesign of the base, a further reduction in number of substrates to accommodate for the substrate length, a mild redesign of the clamp mechanism, and a complete design of the case (Figure 3-3).

In the first two concepts, the base plate fully housed the well plates and was planned to be machined from polytetrafluoroethylene (PTFE). PTFE was selected to minimize friction between the plate and the transverse bars that slid on it. However, because PTFE is a soft material, there were concerns that it would warp over time, especially if placed in an autoclave. Therefore, a thinner stainless steel base was designed instead, with three sliding rails made from PTFE. The rails were bolted to the base plate so even if warping occurred, it would not affect the sliding motion. Slots were also machined on the base plate to fix the locations of the well plates. Four polycarbonate well plates could fit in total, each with two subcompartments. Each
Figure 3-3 – SolidWorks model of final concept in isometric view (a), and top view (b).
subcompartment housed a set of clamps (Figure 3-4) and one substrate, for a total of eight sets of clamps and eight substrates.

![Substrate clamps depicting fixed clamps (a) in open position, (i) and closed position (ii), as well as adjustable clamps (b) in open position, (i) and closed position (ii) (red arrows indicate direction of motion). The fixed clamps were bolted to the fixed transversal bars and the adjustable clamps to the moving transversal bars.](image)

The slots on the transversal rails were replaced with holes to reduce the probability of relative motion during bioreactor operation. The fixed clamps were bolted to the fixed transversal bars and the moving clamps to the moving transversal bars. Each type had a removable bottom portion that defined the way the clamp worked. The substrate was placed between the top and bottom pieces, and the bottom piece was tightened against the substrate using the nuts (Figure 3-4). The moving clamps had an additional degree of freedom due to their slots. The slots were designed to allow for synchronization of prestrain across multiple clamped substrates, if necessary, to ensure the substrates all experienced the same amount of tension before being subjected to the loading profile.

The two fixed transversal bars were each placed at the ends of the well plates to maximize travel distance in the opposite direction. The two moving transversal bars were welded to the main actuating arm, which glided on the middle rail. The final position of the moving bars was calculated to minimize clamping distance and maximize travelling distance. The two linear U-shaped guides were still required to ensure that non-axial movement of the main actuating arm was eliminated. The two guides were made from PTFE to reduce friction. The actuation rod was threaded into the main actuating arm and screwed in after the case was in position (Figure 3-5).
The case was designed from polycarbonate and was assembled by bolting parts together. The top of the case contained two holes for venting cap filter installation. The sleeve bearing was installed to guide the actuating rod out of the bioreactor and was made from PTFE to reduce friction. The case could not be taken off unless the actuation rod was unscrewed. This served as a safety mechanism to prevent accidentally removing the case in a non-sterile environment.

Finally, all parts of the bioreactor except for the linear rails, well plates, sleeve bearing, linear U-shaped guides and the case, were designed to be made out of 303-stainless steel to prevent corrosion.

3.4 Final design
3.4.1 Bioreactor system

The bioreactor was fully manufactured by the University of Toronto Department of Mechanical and Industrial Engineering Machine Shop. Unless otherwise specified, all materials were made from 303-stainless steel and purchased from McMaster-Carr Supply Company (Atlanta, GA, USA). The first version of the final design was identical to the final conceptual design (Figure 3-6). However, testing and troubleshooting of the bioreactor required a few modifications to be made to the first version. All of the subsequent modifications were also performed by the machine shop.
Figure 3-6 – Final version of the bioreactor (case and actuator not pictured) in isometric view (a), and top view (b).
The functionality of the bioreactor was identical to what was described in the final conceptual design. There were four polycarbonate well plates, each with two subcompartments, in which each substrate was clamped. The clamps were bolted to the moving and fixed transversal bars. The fixed bars were bolted down to the side rails, and the moving transversal bars were welded to the main actuating arm, onto which the actuating rod was attached. Dowels on the base were installed in order to secure the case once it was placed in position. The venting caps installed on the case were polypropylene Millipore Millex Vacuum Line Protection and Tank/Bioreactor Venting Filter Units (SLFG75010, Thermo Fisher Scientific; Rochester, NY, USA) with a 0.2 µm pore size PTFE membrane (Figure 3-7).

![Bioreactor venting filters](image)

**Figure 3-7 – Overall bioreactor setup (actuator not pictured).**

The main changes to the first version of the bioreactor were the type of material of the side and middle rails, a redesign of the linear guiding system for the main actuating arm, and a redesign of the actuating rod. A significant problem with the initial system was non-axial movement of the main actuating arm. The linear U-shaped PTFE guides, which were originally installed on the bioreactor, were too soft to counteract transversal forces on the main actuating arm and maintain a straight trajectory. The transversal forces were generated by a large moment created by the nature of the actuation mechanism. Initially, the bioreactor was actuated by a linear actuator that sat parallel to the length of the bioreactor. The bioreactor and the linear actuator were connected by a cross bar that transmitted the motion of the actuator to the bioreactor. A large moment would be created on the cross bar with every stroke of the actuator and this acted as a force...
perpendicular to the actuation rod and the actuation arm. To prevent this problem, aluminum linear motion guide rails were installed and a thicker actuation rod was machined (Figure 3-8). While these features ameliorated the effect of the large moment, there was still a noticeable amount of non-axial displacement. In turn, this caused the actuator to work harder than necessary – on multiple occasions it either stopped actuating after ~20 min or stalled for a few seconds and restarted in the wrong position, thus disrupting the loading pattern. This design was thus abandoned and a new actuation mechanism was implemented (next section). Lastly, the original side and middle rails were manufactured from PTFE due to its low friction properties. However, upon autoclaving, the components had warped and shrunk (contrary to specifications) and could not be screwed to the base plate anymore. A tougher and more heat resistant material called polyether ether ketone was chosen instead. Finally, a stainless steel mounting block was welded to the base plate in order to anchor the new actuation mechanism (Figure 3-6).

3.4.2 Actuation mechanism

In order to mechanically stimulate the substrates clamped inside the bioreactor, an actuation mechanism that provided linear motion was necessary. Initially, the actuation mechanism was a linear actuator that sat parallel to the length of the bioreactor. However, due to aforementioned reasons, this design was abandoned. Ideally, the linear actuator would be in line with the

Figure 3-8 – Photographs depicting the linear guide rail and the guide block (a), and the new actuation rod (b). The actuation rod features are explained later in the context of the actuation mechanism.
actuation rod, thereby avoiding any damaging side loads. This was not possible due to sizing constrains since the setup would not fit in the laboratory incubator. The final choice was a stepper motor connected to a rack and pinion system (Figure 3-9). The gear mounted on the motor’s shaft would act as a pinion and would drive a rack that was connected to the actuating rod. This would minimize the footprint of the actuation mechanism as well as eliminate side loads. Although in this case the only non-axial loads would be forces acting downwards on the actuating rod and creating extra friction on the side rails, friction is easier to overcome with a strong motor than correcting for side loads.

The stepper motor utilized was a high torque NEMA-23 motor with 1.86 Nm of holding torque (HT23-399, Applied Motion; Watsonville, CA, USA). The motor was wired in parallel and was connected to an existing programmable step motor driver (Si2035, Applied Motion; Watsonville, CA, USA) (Figure 3-10). The step motor driver required 110 V DC in order to operate and thus a regular wall plug was wired to supply this power. Motor leads were also wired to the motor driver as well as a computer connection cord that was connected to the PC/MMI input. For ease of transport, the motor drive and computer power supply had been previously attached to a custom acrylic glass base.

In order to take advantage of the existing driver, the motor was selected so as to produce enough torque to stretch eight substrates without applying more than 2 A of current to the driver (maximum allowable limit). According to calculations, the motor had enough torque to stretch
all the required substrates as well as to overcome friction and inertia created by the acceleration of the actuating arm inside the bioreactor (see Appendix A).

![Diagram of motor and step motor driver](image)

**Figure 3-10 – Schematic of the parallel wiring configuration of the stepper motor (a), and the programmable step motor driver (b).**

The rack and pinion (S181YYM0508200 and S10T05M021S0505, respectively, Stock Drive Products/Sterling Instrument; New Hyde Park, NY, USA) were chosen to be 303-stainless steel to prevent corrosion. The pinion was bored to size in order to fit on the motor shaft and was fixed in place with a set screw. To connect the rack to the actuation rod, a tight clearance hole was longitudinally bored into the center of the rod where the rack was inserted. Next, half of a high-pressure compression fitting was welded to the actuation rod and the other half was attached to the rack (Figure 3-8). The compression fitting allowed the rack’s teeth to be oriented in the desired position (regardless of how much the rod was tightened against the actuating arm) and was then tightened. The bored hole was necessary to ensure that the actuation rod and the rack were concentric.

The motor was covered with a 3” diameter heat shrink that conformed to the motor’s profile once heated (HSEC300, Cable Organizer; Fort Lauderdale, FL, USA) and was sealed with a fast set marine grade urethane (Bostik 920-FS, Industrial Supply Group; Marietta, GA, USA). This environmental sealing allowed the stepper motor to function inside the incubator environment. The motor was mounted on an aluminum bracket (MMT 260, CyberResearch; Branford, CT, USA) that was bolted to the baseplate.
3.4.3 Bioreactor system programming (see Appendix B for step-by-step instructions)

A personal computer was connected to the stepper motor driver through an RS-232 PC/MMI connection cord supplied with the motor driver. An adapter cable was used to convert the serial DB9 input of the connection cord into a USB input. The software Si Programmer™ (Applied Motion; Watsonville, CA, USA) was used to program and transmit commands to the step motor driver that would, in turn, control the stepper motor. The program was intuitive to use due to its excellent graphic user interface (Figure 3-11).

Programming of the motor driver consisted of three lines. The first line caused the stepper motor shaft to rotate counter-clockwise by the required amount; the second line rotated the shaft clockwise by the same amount, and the third line was a loop that repeated the process. The rotation speed was adjusted to order to obtain a cyclic displacement at 1 Hz. Furthermore, the acceleration and deceleration was reduced to 5 rev/s² to reduce the impact of high loads due to inertia. The stepper motor was supplied with 0.7 A/Phase (rated at 1.41 A/Phase) to prevent running the motor at its peak and minimize overheating. Although halving the current meant that the torque output was also halved, the stepper motor could produce more than twice the required torque to actuate the substrates so this was not an issue (see Appendix A for torque calculations). To run the motor during an experiment, it was only necessary to supply power to the motor.
driver and the stepper motor would begin its cycle. Connection to a personal computer was thus unnecessary, except during reprogramming.

3.5 Bioreactor calibration

To determine the relationship between motor input and bioreactor output, a calibration curve was created. The curve was generated by instructing the motor to move in 1 mm increments up to 10 mm. Images were captured for every increment and the displacement of the bioreactor’s transversal bar relative to its starting position was measured (Figure 3-12). For consistency, the number of substrates loaded into the bioreactor was the same as the number used in the later biological cyclic experiments.

![Figure 3-12](image)

**Figure 3-12** – Images of the bioreactor moving transversal bar during calibration in starting position at 0 mm (a), and in final position at 10 mm (b).

Each point on the curve was calculated by normalizing the distance the bioreactor moved in one increment to the inputted distance (Figure 3-13).

![Figure 3-13](image)

**Figure 3-13** – Bioreactor calibration curve.
The relationship between bioreactor displacement and inputted distance was linear with a correlation coefficient \( R^2 = 0.9998 \). The trend line equation was subsequently used during experiments to determine the required input in order to achieve the proper bioreactor displacement.

### 3.6 Sterility maintenance and cell viability

The bioreactor was designed to allow for the application of strain, biochemical and matrix stimuli as well as provide an environment that supported cell growth and proliferation. To characterize the bioreactor’s ability to maintain cell viability and sterility, a proof of principle experiment was done whereby PAVICs were cultured in bioreactor versus incubator conditions and their net proliferation rates were assessed.

#### 3.6.1 PAVIC preparation

PAVICs were isolated, according to an established laboratory protocol, from porcine aortic heart valves of pigs that were slaughtered at a local abattoir (Quality Meat Packers; Toronto, ON, Canada). Passage eight cells were used to perform this experiment. Following culture in a standard T75 flask, PAVICs were trypsinized with 0.125% trypsin with EDTA for seven minutes. The cells were counted using a standard haemocytometer and seeded at 20,000 cells/cm² in six standard 35 mm tissue-culture treated polystyrene Petri dishes. Three Petri dishes were placed in the bioreactor and three were placed in the incubator. The cells were cultured for three days in supplemented media (89% Dulbecco’s Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin), which was replaced daily. At day three, the cells in each of the six Petri dishes were trypsinized and counted for comparison.

#### 3.6.2 Statistical analysis

In order to quantify the ability of the bioreactor to maintain cell viability, a Mann-Whitney Rank Sum Test (normality failed) was performed between bioreactor and incubator cell density at day three \( n = 3 \). Results are reported as mean ± standard deviation and in all cases \( p < 0.05 \) was considered statistically significant.
3.6.3 Results and discussion

Cell numbers at day four between incubator and bioreactor conditions were not statistically different \( (p = 0.4) \) (Figure 3-14). This result indicated that total cell number was not statistically affected by the incubator environment and the net proliferation rates in both the incubator and the bioreactor were similar.

![Figure 3-14 – PAVIC cell counts in bioreactor and incubator environment are similar after three days in culture \( (p = 0.4 \) and \( n = 3) \).](image)

Furthermore, sterility maintenance was evaluated by placing media (without cells) in the bioreactor well plates for one week and checking for contamination and color change at the end of the experiment. It was found that media was free of contaminants and its color did not change after one week (results not shown).

3.7 Conclusion

In summary, a versatile bioreactor system was designed that could apply a range of mechanical strains for a fixed displacement (with the option of modifying the loading pattern, if necessary), accommodate substrates of varying geometries and material properties (i.e., substrate stiffness), as well as subject substrates to various biochemical conditions. The bioreactor was integrated and interfaced with an actuating system that transmitted linear motion via a rack and pinion system and a calibration curve was generated. Finally, it was shown that cell cultures inside the
bioreactor could be grown at similar net proliferation rates as those in typical incubator environments. Finally, we demonstrated the maintenance of aseptic conditions for up to one week.
Chapter 4

4 Substrate design and characterization

4.1 Introduction

An important part of this thesis was to design and develop a high-throughput, versatile substrate that was used for cell culture and tissue formation (and retention), could be clamped and stretched in tension by the bioreactor system, and provided strain uniformity. The substrate developed here produced a range of mechanical strains during one stretch cycle, and provided an array of technical replicates for every strain condition, thereby increasing statistical power. The technical replicates represented microwells that were patterned soft lithographically on every strain condition. The microwells were subsequently moulded with hydrogels (Chapter 5) and seeded with PAVICs, and the substrates were clamped in the bioreactor for combinatorial experiments (Chapter 6). The bioreactor applied a cyclical longitudinal displacement to the substrates, and the PAVICs experienced mechanical strain through the hydrogels moulded in the microwells. In this chapter, the focus is on the design, development, and characterization of the substrate and its features (i.e., the patterned microwells).

4.2 Analytical formulation of substrate strains

The substrate was designed to produce different strain magnitudes in five regions along its length. This was achieved via a staircase geometry whereby successively decreasing cross sectional areas produced correspondingly larger strains for a fixed substrate displacement (Figure 4-1). The staircase step thicknesses were derived theoretically through an equation describing their relationship with the desired strain values. To derive the final form of the equation, we started with a linearly elastic rectangular solid, whose stress-strain relationship was given by:

\[ \sigma = E \times \varepsilon \quad (1) \]

where \( \sigma, E \) and \( \varepsilon \) were the engineering stress, Young’s modulus, and engineering strain, respectively. The engineering stress could also be written as:

\[ \sigma = \frac{F}{t \times w} \quad (2) \]
where $F$, $t$ and $w$ were the applied longitudinal tensile force, thickness, and width of the solid, respectively. Substituting (2) into (1), and solving for $F$, the following was obtained:

$$F = E \times \varepsilon \times t \times w \quad (3)$$

A rectangular solid with uniform width and multiple step thicknesses experiences the same applied force in every step region. Therefore, by equating the force in each region, an equation describing the relationship between all the strains and thicknesses ($w$ and $E$ cancelled out because of constancy) was obtained:

$$\varepsilon_1 \times t_1 = \varepsilon_2 \times t_2 = \varepsilon_3 \times t_3 = \varepsilon_4 \times t_4 = \varepsilon_5 \times t_5 \quad (4)$$

where the subscripts indicated the step number. The values of the desired strains and the minimum step thickness were used as inputs to calculate the thicknesses of the remaining steps (Figure 4); to minimize stress concentrations, corners between steps were filleted with a 1/16” radius. Once the step thicknesses were computed, the width of the substrate was determined such that it would fit comfortably in the bioreactor clamps (Figure 4-2a). The nature of the clamping mechanism required a portion of the substrate to sit behind the clamp during stretching. The ends of the substrate were thus designed appropriately (Figure 4-2b).

The nominal strain magnitudes of the substrate were finalized as 2.5%, 5%, 10%, 15%, and 20% on the 8 mm, 4 mm, 2 mm, 1.33 mm, and 1 mm step thicknesses, respectively.
4.3 Microwell design

An array of microwells in the shape of dog-bones was designed for soft lithographical patterning on the substrate. The dog-bone shape is a standard geometry widely used in tensile testing; this is due to the uniform strains it exhibits along the straight section during stretch. Accordingly, we incorporated the dog-bone geometry into our design. Each dog-bone microwell featured a sizeable straight section, and an “ear” section at each end (Figure 4-2a, inset). Therefore, the cell-seeded hydrogel material that would later be moulded in every microwell would take the shape of a dog-bone. It was hypothesized and later confirmed (Chapter 5) that the hydrogel would exhibit strain uniformity along its straight section similar to a dog-bone undergoing tensile testing. This was a desirable outcome since cells growing in that region would be subjected to uniform tissue or biomaterial strain.
The array consisted of seven parallel microwells laid along each step region of the substrate (35 total per substrate) (Figure 4-2a). The microwells were patterned towards the center of each step region to avoid strain non-uniformities due to the edge of the substrate and the step change in substrate thickness.

4.4 Substrate fabrication

The need to integrate hydrogel materials with cell cultures on a deformable, transparent, and biocompatible substrate immediately suggested the use of polydimethylsiloxane (PDMS), a curable elastomer used ubiquitously in microfabricated systems. All of the staircase substrates and additional components thereof were thus fabricated exclusively from PDMS.

4.4.1 Staircase substrate

The substrate was fabricated from Sylgard 184 PDMS (Dow Corning through Ellsworth Adhesives Canada; Burlington, ON, Canada) through a combination of soft lithography techniques [140] and a modified squeeze fabrication procedure [141]. Two moulds were used: (1) A 303-stainless steel mould machined by the University of Toronto Department of Mechanical and Industrial Engineering Machine Shop was used to produce the staircase geometry of the substrate (Figure 4-3). The mould had two holes in its bottom to allow the
substrate to be pushed out after curing; (2) Microfabricated SU-8 masters were used to pattern the dog-bone shaped microwells by replica moulding.

To create the SU-8 masters, a series of steps were undertaken (see Appendix C for step by step protocol). First, a seed layer that increased the adhesion of features to glass was fabricated by spin-coating SU-8 5 (Microchem; Newton, MA, USA) at 3000 RPM on 3” x 2” glass slides. The slides were pre-baked, exposed to UV light, and post-baked to obtain a seed layer 7 µm thin. To fabricate the dog-bone features, SU-8 50 (Microchem; Newton, MA, USA) was spin-coated at 1000 RPM on the seed layer glass slides, after which the slides were pre-baked, exposed to UV light through a photomask (printed at CAD/Art Services, Inc.; Bandon, OR, USA), and post-baked to obtain a thickness of 150 µm. The process was repeated to add a second layer of SU-8 50 after which the slides were developed and hard-baked achieving a final feature thickness of 300 µm (the depth of the microwells). Before using the SU-8 masters for replica moulding, a silanization treatment was performed in order to prevent PDMS from sticking to the surface and delaminating the features. The masters were treated with the silanization agent (Tridecafluoro-1,1,2,2-tetra-hydrooctyl)-1-trichlorosilane (United Chemical Technologies; Bristol, PA, USA), under vacuum conditions [142].

The substrates were fabricated as described below and in Figure 4-4. First, the stainless steel mould was thoroughly cleaned with water, soap, and isopropanol (IPA), after which it was dried using nitrogen gas. The mould was then placed on top of a polyethylene film (Ink-jet transparency, Grand & Toy; Toronto, ON, Canada) sitting on a polycarbonate plate. Before placing the mould on the plate, the plate was thoroughly wiped with tissues doused in IPA and dried with nitrogen gas. PDMS, mixed in a standard 10:1 curing ratio, was poured into the mould such that the PDMS overflowed a little in order to cover the edges of the mould. In parallel, 2.5 mL of PDMS was poured on the surface of the SU-8 master to coat it fully. Both moulds were placed in a desiccator to remove trapped air bubbles; in some cases, bubbles remaining in the ends of the dog-bone features after desiccation were burst manually using a 20-gauge needle. The SU-8 master was then turned upside down and carefully placed on top of the substrate mould to avoid introducing air at the PDMS:PDMS interface. Since the SU-8 master did not entirely cover the whole area of the substrate mould, a silanized glass slide was added at each end of the SU-8 master. Another polyethylene film was added on top, followed by another polycarbonate well plate. The construct was clamped and cured at 80 °C overnight. Extensive
experimentation with the moulding of dog-bone shaped hydrogels into the staircase substrate microwells showed that the hydrogels would freely float away from their moulds once the substrate was submerged into cell media. This demonstrated the need for a “trapping”
mechanism to ensure the hydrogels remained in their appropriate locations during cell culture and stretching. Measures such as chemical treatment of PDMS to bond the hydrogel, modification of the SU-8 masters, or creation of epoxy masters to add overhangs in the dog-bone features, were all attempted but were unsuccessful. The final working method was the fabrication of a thin PDMS membrane containing the outlines of the dog-bone microwell pattern, inset by 250 µm. The thickness of the membrane was 120 µm, selected to minimize alteration of the strains of the substrate steps. The membrane features were carefully aligned with the substrate features and the membrane was plasma bonded to the substrate. Since the membrane created a 250 µm overhang covering the initial dimensions of the microwell, the hydrogels were immobilized within their moulds. The PDMS membrane was fabricated through a squeeze fabrication procedure previously demonstrated in our lab [141]. Briefly, an SU-8 master (fabricated as described above via one spin coating step) containing the desired features was coated with 2.5 mL of PDMS. The master was placed on top of a piece of foam that sat on a polycarbonate plate. A transparency was carefully laid on top of the coated SU-8 master without introducing any bubbles. A 3” x 2” silanized glass slide was placed over the transparency to distribute the pressure evenly. Another piece of foam was placed on top, a second polycarbonate was added and the construct was clamped and placed in an 80 °C oven overnight. The purpose of the foam was for cushioning.

To prepare for bonding, the membrane attached to the transparency was removed from its mould; the polycarbonate well plates, polyethylene films, SU-8 master and silanized glass slides were removed from the staircase substrate, but the substrate was left in its mould to avoid misalignment-induced shrinkage. Both the substrate and the membrane were oxygen plasma treated using a plasma cleaner (Model PDC-001, Harrick Plasma; Ithaca, NY, USA) for three minutes. Next, four PTFE spacers (McMaster-Carr Supply Company; Atlanta, GA, USA) were placed on the edges of the stainless steel mould (two on each side) and the transparency containing the membrane was positioned on top of the spacers. Using a light stereoscope (Model SZ61, Olympus Canada Inc.; Richmond Hill, ON, Canada), the membrane features were aligned with the substrate features. Once aligned, the transparency was gently pushed downward until it contacted the staircase substrate surface. The ensemble was placed in an 80 °C oven for 10 minutes to finalize the bonding. Finally, the transparency was gently lifted, the patterned staircase substrate was removed from the mould, excess PDMS was trimmed manually, and
PDMS blocks were bonded to the ends to enable clamping in the bioreactor, as previously mentioned (Figure 4-5).

**Figure 4-5 – Photograph of the finished PDMS staircase substrate clamped in the bioreactor for stretching.**

4.4.2 Static substrates

For non-stretched (static) control samples, flat substrates patterned with dog-bone microwells were fabricated following the procedure in the previous section, but by using an Omniwell tray lid (Nunc through Thermo Fisher Scientific; Rochester, NY, USA) instead of the staircase mould to form the base.

4.5 Substrate strain characterization

To characterize the strain distribution within the substrate steps, a series of analyses and measurements was performed. A finite element analysis (FEA) was first run on a virtual model of the substrate, after which empirical measurements of the dog-bone microwell features were taken and compared to the FEA results.

4.5.1 Finite element analysis

As part of the substrate design process, FEA was used in conjunction with the analytical model to estimate the strains generated in the substrates during stretching. A 3D model generated using SolidWorks 2010 (Dassault Systèmes SolidWorks Corp.; Waltham, MA, USA) was analyzed by
the FE method using ANSYS 13.0 (ANSYS, Inc.; Canonsburg, PA, USA). The model included the added membrane thickness and represented the substrate without the microwells to save computational power. The model was modeled with a large displacement linear elastic material using the 20-node solid element SOLID186. Displacement boundary conditions were applied as in the bioreactor: the fixed clamp end was constrained from movement in all directions, and the moving clamp end was subjected to axial x-direction displacement, but constrained in the orthogonal directions (Figure 4-6). Plots showing the initial and deformed shapes of the substrate (Figure 4-7) as well as engineering axial nodal elastic substrate strain (along the x direction) were generated (Figure 4-8). The axial nodal elastic substrate strain plot (Figure 4-8) demonstrates qualitatively that each step in the staircase substrate displayed approximately discrete strain profiles.

Figure 4-6 – Screen captures from ANSYS showing the FE model and meshing in isometric view (a), and side view (b). The mesh was refined on the top surface of the substrate to improve accuracy. Arrows in (b) depict the surfaces of the substrate that were constrained during the simulation (A, B, C, D were constrained in all directions; E and F were displaced by 7.0231 mm in the positive x direction; G and H were constrained constrained in the y and z directions; for clarity, red arrows depict the surfaces where displacement was applied; Cartesian coordinates are shown for reference).

To quantify the axial strains exhibited by the substrate at the microwell locations and compare them with analytical values, the locations of keypoints assigned to the center of every dog-bone
Figure 4-7 – Schematics showing the deformed (solid line) and undeformed (dashed line) staircase substrate in side view (a), and top view (b) (Cartesian coordinates are shown for reference; red arrows indicate direction of motion).

Figure 4-8 – Plot showing the engineering axial nodal elastic substrate strain distribution. It can be noticed that the different step regions exhibit approximately discrete strain profiles (Cartesian coordinates are shown for reference).
microwell ear post were recorded before and after stretch. Using the ear post positions, engineering axial substrate strain values were generated at the position where each dog-bone microwell would sit in the array. These strain values were averaged across the seven microwell locations for every substrate step in the staircase, and compared with analytical values.

There was generally good agreement between the theoretical values and the FEA predictions (Figure 4-9)

![Graph showing analytical strains versus average substrate axial strains at the microwell locations on each substrate thickness. A relatively close correlation is observed. Results are presented as mean ± standard deviation.](image)

Figure 4-9 – Results showing analytical strains versus average substrate axial strains at the microwell locations on each substrate thickness. A relatively close correlation is observed. Results are presented as mean ± standard deviation.

The FEA average substrate strains at the microwell locations exhibited some variability (as indicated by the standard deviation in Figure 4-9) on each substrate step since not all the individual strains at the microwell locations were equal. Because the analytical results have intrinsically no variability, a quantitative assessment of whether a statistical difference exists between the analytical and FEA results could not be calculated. The means of the analytical strains were greater than the FEA strains by ~4%, 15%, 6% and ~5% on the 4 mm, 2 mm, 1.33
mm, and 1 mm substrate thicknesses. On the 8 mm substrate thickness, the FEA strain was greater by ~46%.

Although the correlation is fairly close, there do appear to be some differences. First of all, in the analytical formulation, the 100 μm height of the membrane was not considered but its effect is suspected to be small. Furthermore, the analytical formulation by nature predicts strains averaged over the entire step regions that exhibit step-wise changes at the step edges. However, this is not an accurate representation of how the strain is distributed throughout the physical model or the real substrate. In reality, the substrate experiences a gradient of strain, and the approximately discrete strain profiles on the substrate steps slightly overlap with adjacent steps (Figure 4-8). Therefore, the average values predicted by the analytical formulation are not maintained. The effect of the filleted corners between steps was also not included in the analytical formulation. These reasons are most likely the cause of the discrepancy between the FEA and the analytical data and although there are some differences, they appear to be small (except on the 8mm thickness).

4.5.2 Experimental measurements

The analytical and FEA estimates of substrate strains were also validated experimentally. To facilitate higher-resolution imaging than was possible in the bioreactor, the substrates were stretched using a custom designed single-substrate stretching device (Figure 4-10). The device consisted of a left-right screw motion mechanism with attached steel clamps similar to those in the bioreactor. Staircase substrates were prepared as outlined previously. Although this experiment involved collecting measurements for hydrogel and substrate strains, this chapter focuses only on the substrate measurements.

The substrates were clamped in the stretch device and photographed in the unstretched and stretched positions using a Navitar high magnification (12x) zoom lens (Navitar; Rochester, NY, USA) and a high resolution IEEE1394 digital black & white camera (Sony; Vienna, Austria). Images were analyzed with ImageJ (NIH) to calculate strains in the microwells corresponding to different nominal strain magnitudes. Microwell longitudinal (axial) strain was determined from the relative displacement of the centers of the dog-bone microwell posts from the unstretched to the stretched position.
To evaluate the validity of the FEA predictions, empirical microwell axial strain values were averaged in each step ($n = 7$) and across multiple substrates ($n = 6$), and were compared to FEA average axial substrate step strains at the microwell locations ($n = 7$) through student t-tests or Mann-Whitney Rank Sum tests, if equal variance failed. To quantify the variation in empirical axial microwell strain magnitudes in one substrate step and across steps, two-way ANOVA and post-hoc Holm-Sidak pairwise comparisons were performed. Results are reported as mean ± standard deviation and in all cases $p < 0.05$ was considered statistically significant.

For the same step region, average empirical microwell strains and average FEA substrate strains at the microwell locations were statistically different in most cases (Figure 4-11). The means of the FEA strains were greater than the empirical means by $18\%$, $15\%$, and $2.5\%$ (negligible) on the 8 mm, 4 mm, and 2 mm steps. Conversely, the mean empirical strains were greater than the mean FEA strains by $3.4\%$ on the 1 mm condition (negligible). The observed differences could have arisen from consistently slightly inaccurate measurements in ImageJ or improper loading of the substrates in the stretching device. Additionally, the geometry of the substrates could not be fully reproduced in ANSYS (i.e., all the fillets).
In the two-way ANOVA test, pairwise comparisons showed no significant difference between dog-bone locations within a step region, except between positions one and two (p < 0.05). However, the difference in means was 3.6% and was thus considered negligibly small.

Furthermore, axial microwell strain magnitudes were statistically different between substrate step regions and this was a desirable outcome (Figure 4-12). Strains measured in the same position on different substrates were in close agreement as represented by the small sample deviation (coefficient of variation ranged from 0.25% (location two on the 8 mm thickness) to ~13% (location seven on the 8 mm thickness) but most were less than 4%). This signified small substrate-to-substrate variability, which was another desirable outcome.

**Figure 4-11 – Results depicting the average empirical microwell axial strains versus average FEA substrate axial strains at the microwell locations on each substrate thickness. Mann-Whitney Rank Sum Tests or student t-tests showed statistical differences between the average strains of the two conditions on the 8 mm, 4 mm, 2 mm, and 1 mm substrates. Results are presented as mean ± standard deviation (**p < 0.001 and n = 6 substrates on the empirical condition).**
4.6 Conclusion

In summary, a flexible substrate that could be clamped and stretched in the bioreactor was designed. By modifying the substrate geometry, the substrate was designed to exhibit five different strain profiles along its length. An array of dog-bone shaped microwells was patterned on the surface of the substrate and FEA analyses confirmed that substrate strains at the microwell locations were in relatively close agreement to analytical values. FEA substrate strains at the microwell locations were also compared to empirically measured microwell strains and the values were in close agreement. The substrate displayed low variability across multiple samples.
Chapter 5

5 Hydrogel system integration and characterization

5.1 Introduction

The selection of a suitable hydrogel system that was easily integrated with the bioreactor system, more specifically with the staircase substrate, was an important part of this study. The staircase substrate contained seven micropatterned dog-bone shaped microwells on five regions along its length, with each region straining a different amount during stretch. In this chapter, the focus is on the selection of an appropriate hydrogel system, followed by the fabrication and characterization of the hydrogel in the staircase substrate.

5.2 Hydrogel selection

5.2.1 Collagen

Due to its physiological relevance and abundance in the aortic heart valve [38], collagen was the first choice as the potential hydrogel system. Extensive work has been performed with cell-seeded or cell-laden collagen gels [83-91, 103, 104], and a more recent study by Legant et al. [84] measured cell-generated traction forces within 3D fibroblast seeded collagen gels bound between two flexible cantilevers. Collagen is therefore a well-established and suitable system for microtissue engineering.

5.2.1.1 Fabrication and integration into substrate

Collagen solution was prepared by combining 10X concentrated DMEM, 0.25 M NaHCO₃ buffer, FBS, Penicillin/Streptomycin, 0.01 M NaOH, and 97% Type I bovine collagen (Advanced Biomatrix; San Diego, CA, USA) in a ratio of 1:1:1:1:0.5:8, respectively. Stock concentrations of collagen used were 4 mg/mL and 6 mg/mL. After dilution, the final collagen concentrations were 2.62 mg/mL and 3.93 mg/mL, respectively.

To test and optimize the moulding of the gels in the dog-bone shaped microwells, flat substrates were used initially, since they were more easily fabricated than staircase substrates (Chapter 4, Sections 4.4.1 and 4.4.2). The final protocol for moulding the gels was not expected to change with the substrate geometry. Before adding the collagen solution to the flat substrates, the
substrates were processed to prevent the formed dog-bone gel from floating away once in solution. This involved placing PDMS droplets on top of the microwell posts (Figure 5-1). The droplets were deposited one by one using a 20-gauge needle dipped in uncured PDMS and a light stereoscope (Model SZ61, Olympus Canada Inc.; Richmond Hill, ON, Canada). Surface tension prevented the droplets from spilling into the microwells. Once all the posts were processed, the substrate was placed in an 80 °C oven for four hours to allow the droplets to cure. The diameter of the droplets was slightly larger than the post diameter and this trapped the dog-bone gels inside the microwell.

Once the droplets were cured, the substrate was oxygen plasma treated with a plasma cleaner (Model PDC-001, Harrick Plasma; Ithaca, NY, USA) for three minutes to increase the surface hydrophilicity and allow the collagen solution to flow into the microwells. The surface of the substrate was covered with collagen solution until all the microwells were filled. Excess collagen residing outside of the microwells was aspirated using a glass Pasteur pipette. The substrate was then placed in a laboratory incubator at 37 °C for one to two hours for polymerization to occur. In order to embed fibroblasts in the gel, the collagen solution was mixed with PAVICs (see Chapter 6, Section 6.2 for cell preparation procedure) suspended in a 15 mL centrifuge tube with the supernatant solution removed.

On the static substrates, although the dog-bones without cells exhibited good mechanical integrity (Figure 5-2a), the cell embedded dog-bones were mildly remodeled after 24 hours and more extensively after four days in culture (Figure 5-2b, c). The remodeling was exhibited in the form of necking in the straight section of the gel. The cells aligned longitudinally as the
remodeling progressed, and the tension of the collagen gel increased. Most importantly, the increase in tension of the gels combined with external strain from the staircase substrate caused all the gels to rupture during cyclical stretching, irrespective of the collagen concentration (Figure 5-2d).

In summary, while collagen hydrogels exhibited good mechanical integrity without cells, following cell embedding, the static gels remodeled, which increased their tension. During stretching, all the dog-bone gels failed because of the combined tension and external strain. It was therefore necessary to select an alternative hydrogel system that was more mechanically robust.

Figure 5-2 – Images depicting collagen gels without cells (a), PAVICs embedded in collagen gels after 24 hours (b), PAVICs embedded in collagen after four days of culture (c), and ruptured gels after 24 hour stretch (d) (arrows indicate gel edges).
5.2.2 Gelatin methacrylate (gelMA)

GelMA is a photopolymerizable hydrogel composed of methacrylate pendant groups that are bound to a heterogeneous mixture of water-soluble proteins found in collagen [108]. Gelatin, which is a denatured form of collagen that still maintains cell-binding motifs, can be made light polymerizable by the addition of methacrylate groups to its amine-containing groups [107]. GelMA has been shown to support adhesion, proliferation and organization of cell cultures, exhibit excellent micropatterning properties, and be easily tailored to different stiffnesses [105, 108-110], making it an attractive candidate for creating cell-seeded microtissues, and a suitable choice for this study.

5.2.2.1 Fabrication and integration into substrate

GelMA was synthesized as described previously [108]. To reiterate, gelatin from porcine skin (Sigma-Aldrich Canada Ltd.; Oakville, ON, Canada) was dissolved in phosphate buffered saline (PBS) without Ca\(^{2+}/Mg^{2+}\) at 50 °C and stirred for 30 minutes until the solution became clear, ensuring little foaming or bubbles occurred in the process. Methacrylic anhydride (94%) (Sigma-Aldrich Canada Ltd.; Oakville, ON, Canada) at 8% (v/v) was added to the solution at a rate of 0.5 mL/min under stirred conditions and allowed to react for three hours at 50 °C. The pH of the solution was monitored throughout and was kept at pH = 5 by adding a few drops of 5 M NaOH solution, when necessary. This step was critical in ensuring the solution did not clump into a milky and viscous fluid. The reaction was stopped after a 3x dilution and the solution was poured into dialysis tubing (S432706, 08-667E, SpectrumLabs through Thermo Fisher Scientific; Rochester, NY, USA) and dialyzed against deionized water at 50 °C for one week. The solution was then filtered and lyophilized for another week. The remaining product was a white foam that could be stored at room temperature in a dry environment and was the precursor for the hydrogel.

To create the final crosslinked form of the hydrogel, the precursor was mixed with a photoinitiator and exposed to UV light for a set amount of time. Briefly, Irgacure 2959 (2-hydroxy-1-(4-(hydroxyethoxy)phenyl)-2-methyl-1-propanone, Ciba Specialty Chemicals, Inc.; Basel, Switzerland) was used as a photoinitiator and dissolved at 2% (w/v) concentration in PBS at 80 °C for 20 minutes. The foam precursor was then dissolved in the photoinitiator solution at 80 °C for 20 minutes. Changing the weight/volume percentage of the foam precursor in the
photoinitiator solution produced hydrogels of varying stiffnesses. Increasing the percentage would result in a stiffer gel and vice versa (range achieved estimated to be 2.5 kPa to ~43 kPa).

In parallel, staircase PDMS substrates (Chapter 4) were prepared by oxygen plasma treating the surface for three minutes to allow the uncrosslinked solution to flow into the dog-bone microwells. Once dissolved, the uncrosslinked solution was poured over the empty microwells until all were filled. A 7.4 cm x 5.5 cm perfluoroalkoxy (0.005” thin) (McMaster-Carr Supply Company; Atlanta, GA, USA) film was gently laid onto the surface of the substrate, forcing out any excess gelMA that had not filled the microwells; a blotting roller was used to apply uniform pressure. To prevent it from deforming during rolling, the substrate was placed into its stainless steel mould and this kept it horizontal. The ensemble was placed under a Blak-Ray long wave UV lamp (Model B 100AP, UVP; Upland, CA, USA) and exposed according to previously optimized exposure times (the required UV exposure times used for 5, 8.5, 10, 15, and 20 wt% gelMA precursor in 2% photoinitiator solution were 240 s, 225 s, 180 s, 165 s, and 150 s, respectively). The measured intensity of the lamp was ~11.5 mW/cm² at a distance of ~4.5 cm from the bulb. The film was removed by sliding it off the substrate while submerged in a sterile PBS bath. The substrates were then either seeded with PAVICs (Chapter 6) or stored in PBS for strain characterization studies.

5.3 Hydrogel strain characterization

As outlined previously (Chapter 4, Section 4.5.2), gelMA moulded staircase PDMS substrates were clamped in a stretch device and photographed in the unstretched and stretched positions (Figure 5-3). In order to track the strain of the gels, tissue-marking dye (Triangle Biomedical Sciences, Inc.; Durham, NC, USA) was dotted on the surface of the gels using sharpened toothpicks. Before imaging, the gels were placed in a state of prestrain by introducing a minimal displacement on the staircase substrate. This ensured that the ears of the gels were in contact with the PDMS posts and that they would start stretching as soon as more straining was introduced. The gels were hydrated with PBS throughout the experiment. Hydrogel axial engineering strains were determined from the relative displacement of pairs of dots (D2 and D6 on Figure 5-3) on the hydrogels from the unstretched to stretched position. Measurements were confined to the middle “shaft” region of the dog bone-shaped samples, where strains were predicted to be uniform. To quantify strain uniformity, higher resolution images were taken of
the dots (D1 through D6 on Figure 5-3); axial engineering strains for each dot along the shaft region were calculated using its change in length from the unstretched to stretched position. The final measurements were performed on substrates moulded with hydrogels of two stiffnesses (8.5 wt% corresponding to ~12 kPa, and 20 wt% corresponding to ~43 kPa), with three substrates per stiffness value.

**Figure 5-3** – Dog-bone shaped hydrogels in the unstretched (a), and stretched (b) configuration (arrows depict the locations of the six ink dots on (a), and three of seven hydrogels on (b) – positions 4 through 7 not pictured).

### 5.3.1 Statistical analysis

To evaluate the axial strain magnitudes of the hydrogels within a substrate step, across steps, and between two different hydrogel stiffnesses, three-way ANOVA and post-hoc Holm-Sidak pairwise comparisons were performed and to further evaluate the data, student t-tests were performed (n = 3 substrates per stiffness value). Furthermore, paired student t-tests were performed between step regions to compare the axial strains of the hydrogels to those of the PDMS microwells in which they had been moulded (n = 3 substrates per stiffness value). To evaluate strain uniformity in the straight section of hydrogels, along hydrogels in the same step region, and across substrate steps, for both stiffnesses, three-way ANOVA and post-hoc Holm-Sidak pairwise comparisons were performed (n = 3 substrates per stiffness) on the axial strains of each of the dots. Results are reported as mean ± standard deviation and in all cases p < 0.05 was considered statistically significant.
5.3.2 Results and discussion

For all hydrogel measurements, the 20% nominal strain region was not analyzed because the ears of the hydrogels either failed or slipped over the PDMS posts on almost all substrates. Therefore, the gels in this region exhibited much lower strain values than they would have, had they remained intact.

For both stiffness values (8.5 wt% and 20 wt%), axial hydrogel strains were not statistically different (p = 0.62) from all other hydrogels within one step region. However, each step region was statistically different (p < 0.001) from all other step regions (Figures 5-4 and 5-5). Both results were desirable and were similar to the results obtained in the staircase substrate characterization (Chapter 4). The two stiffness conditions were statistically different from each other with p < 0.001.

![Graph](image)

Figure 5-4 – Results comparing axial hydrogel strains within one substrate step and across steps for the 8.5 wt% stiffness (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Three-way ANOVA revealed that hydrogel strains across substrate steps were statistically different. Results are presented as mean ± standard deviation (**p < 0.001 and n = 3 substrates per stiffness).
During tissue dye marking, it was noticeable that the 8.5 wt% gel was stickier than the 20 wt% gel. An increase in stickiness could have resulted in an increase in adhesion to the PDMS substrate, while the 20 wt% gel may not have adhered to the same extent. Different degrees of adhesion would have caused slippage/debonding of the gels during stretch, especially in the higher strain regions. To elucidate this further, student t-tests were performed on the hydrogel axial strain values between nominal strain regions (i.e., substrate steps) on the two stiffness conditions. The means of the averaged axial strain values between the two conditions were statistically different in two cases, the 2.5% and 15% nominal strain regions ($p < 0.001$ in both cases). The mean strain levels of the 8.5 wt% gels were greater by ~18% and ~3.8% compared to the 20 wt% gels on the 2.5% and 15% nominal strain regions, respectively (Figure 5-6). The $p$ values for 5% and 10% nominal strain regions were $p = 0.11$, $p = 0.278$, respectively. Since the difference between means on the 15% nominal strain level was small it was not considered to be relevant. In the 2.5% nominal strain case, the discrepancy could be explained through the fact

**Figure 5-5 – Results comparing axial hydrogel strains within one substrate step and across steps for the 20 wt% stiffness (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively).** Three-way ANOVA revealed that hydrogel strains across substrate steps were statistically different. Results are presented as mean ± standard deviation (**$p < 0.001$ and $n = 3$ substrates per stiffness).**
that the ears of the 20 wt% hydrogels may still not have been in contact with the PDMS posts even after prestraining. This would have caused the hydrogels to reach a lower state of strain at maximal stretch.

Figure 5-6 – Results representing average hydrogel step strains across nominal strain levels, for the 8.5 wt% and 20 wt% stiffnesses (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Student t-tests showed that the mean strain levels on the 2.5% and 15% conditions were statistically different between the two stiffnesses (*p < 0.001 and n = 3 substrates per stiffness).

Hydrogel strains in every step region were also compared to PDMS strains at the microwells where the hydrogels had been moulded. The hydrogel strains were statistically different from the PDMS strains across almost all step regions and in both stiffness conditions (Figures 5-7 and 5-8). In both cases, it is noticeable that the difference between PDMS and hydrogel strains increases with decreasing substrate thickness, which correlates to an increase in strain. In the 8.5 wt% stiffness condition, the PDMS average step strains were greater than hydrogel average step strains by 1.09%, 7.86%, 11.05%, and 18.12%, for 8 mm, 4 mm, 2 mm, and 1.33 mm substrate thickness, respectively. In the 20 wt% stiffness condition, the means were greater by 10.16%,
10.63%, 9.3%, and 20.71%, respectively. The origin of this gradual discrepancy may be twofold. Firstly, as the substrates stretch to the maximal position for the first time, they may slip/detach from the bottom of the PDMS microwell and thus experience lower strains throughout the stretching cycle. When the hydrogel ears delaminate, they snap against the PDMS posts, thereby losing a portion of the strain.

![Graph comparing average step strains of the 8.5 wt% stiffness hydrogels with their PDMS counterpart across substrate steps (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Paired student t-tests revealed statistical differences for the 4 mm, 2 mm and 1.33 mm substrate step thicknesses (*p < 0.05, **p < 0.001 and n = 3 substrates per stiffness).](image)

Furthermore, if the gels fully delaminated and were held in place only by the tension generated due to stretch, their ears most likely sustained a higher strain than the straight section, thereby reducing the strain experienced in the straight region. Based on solid mechanics theory, a narrower cross-section (the ear) stretches more for the same applied force than a wider cross section (the straight section). Although not quantitatively measured, this finding was observed qualitatively in some cases during imaging (Figure 5-9).
Figure 5-8 – Results comparing average step strains of the 20 wt% stiffness hydrogels with their PDMS counterpart across substrate steps (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Paired student t-tests revealed statistical differences for the 8 mm, 4 mm, 2 mm and 1.33 mm substrate step thicknesses (*p < 0.05, **p < 0.001 and n = 3 substrates per stiffness).

Figure 5-9 – Images depicting no slip (a), and slip (b) conditions while the hydrogels are stretched. It is noticeable that the edge of the hydrogel is equally spaced from the edge of the PDMS post on the no slip condition (a), but partially coincides with the edge of the PDMS post on the slip condition (b), denoting loss of adhesion from the PDMS substrate underneath.
Correlation curves between average hydrogel step strains and average PDMS step strains were created to demonstrate that although the magnitude of strain varied, there was a high degree of linear correlation as given by the linear correlation coefficients of $R^2 = 0.9924$ and $R^2 = 0.9907$ for 8.5 wt% and 20 wt% stiffnesses, respectively (Figure 5-10). Using the linear curves, it was possible to predict average hydrogel step strains for a given average PDMS step strain. These calibration curves enabled the hydrogel strains to be predicted by given PDMS strains and demonstrated that the contribution from the confounding factors scaled linearly.

![Linear correlation curves between average hydrogel step strains and average PDMS step strains for the 8.5 wt% stiffness condition (a), and the 20 wt% stiffness condition (b) (trendline equations indicated on figure) (n = 3 substrates per stiffness).](image)

To evaluate strain uniformity in the straight sections of the hydrogels, engineering axial strains for six different dots (Figure 5-3a) marked along the straight portion of the hydrogels were compared within each hydrogel, across hydrogels on one step, and across steps. In the 8.5 wt% stiffness condition, there were no statistical differences between dot strains on individual hydrogels or between different hydrogels in the same step. As expected, strains across different steps were statistically different with $p < 0.001$ (Figure 5-11). Similarly, in the 20 wt% stiffness condition, dot strains across different steps were statistically different with $p < 0.001$ (Figure 5-12). However, there were statistical differences between dots 6 and 2, and 4 and 2, with $p < 0.001$ in both cases. Nevertheless, the associated mean differences were between the two pairs of dots conditions were ~7% and ~5%, respectively, and were therefore considered negligible. Lastly, the strains of hydrogels in position 3 were statistically different from strains of hydrogels in position 1, with $p < 0.001$ (see Figures 5-4 and 5-5 for assigned hydrogel positions) but the
Figure 5-11 – Results comparing average axial dot strains within one substrate step and across steps for the 8.5 wt% stiffness (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Three-way ANOVA revealed that dot strains across substrate steps were statistically different. Results are presented as mean ± standard deviation (**p < 0.001 and n = 3 substrates per stiffness).

Figure 5-12 – Results comparing average axial dot strains within one substrate step and across steps for the 20 wt% stiffness (8 mm, 4 mm, 2 mm, and 1.33 mm substrate thicknesses correspond to 2.5%, 5%, 10%, and 15% nominal strain levels, respectively). Three-way ANOVA revealed that dot strains across substrate steps were statistically different. Results are presented as mean ± standard deviation (**p < 0.001 and n = 3 substrates per stiffness).
difference in means was only 4.6%, and was thus considered negligible. In part, while not large, these differences may have arisen from various degrees of slipping/debonding of the gels from the bottom of the PDMS moulds during stretch since 20 wt% hydrogels were less sticky than 8.5 wt% gels. However, in both stiffness conditions strain uniformity was largely present and this was a favorable result that supported the dog-bone shape solid mechanics theory.

5.4 Conclusion

In summary, gelatin methacrylate was selected as an appropriate hydrogel system that supported growth, adhesion and proliferation of cell cultures, exhibited excellent micropatterning properties, and was easily tunable to varying stiffness values. A fabrication protocol was developed and UV exposure times were optimized to produce mechanically integral gels. Upon strain characterization, it was found that hydrogel strains were statistically different on different gel stiffnesses and from the strains of the PDMS microwells in which the gels had been moulded. These discrepancies were attributed to the possible loss of adhesion of the gel from the bottom of the PDMS mould coupled with the fact that during stretch, the dog-bone gel ears carried a larger portion of the strain compared to the straight section. Furthermore, strain uniformity analysis revealed that the gels experienced largely uniform strain across their straight section, on both stiffnesses.
Chapter 6

6 Cellular and bioreactor studies

6.1 Introduction

The final aim of the thesis was to subject the cell-laden microfabricated substrates described before (Chapters 4 and 5) to a range of uniform tensile strains using the bioreactor system we developed. Since most of the work went into designing, building, and troubleshooting the bioreactor and its actuation mechanism, designing and fabricating the staircase substrates, and incorporating a hydrogel system into the substrates, the stretching experiments served as proof-of-concept to demonstrate the utility of the overall system. To briefly reiterate the procedure, microfabricated substrates that contained regions straining different amounts during stretch were moulded with hydrogels, seeded on top with PAVICs, and subjected to cyclic tension for a given period to assess the expression of $\alpha$-SMA, a marker that indicates the differentiation from the quiescent fibroblast phenotype into the active, contractile, and often disease-associated myofibroblast. This chapter presents the initial cellular experiments in preparation for the cyclic study, as well as the cyclic study itself.

6.2 Cell source

PAVICs were isolated, according to an established laboratory protocol, from porcine aortic heart valves of pigs that were slaughtered at a local abattoir (Quality Meat Packers; Toronto, ON, Canada). Unless otherwise specified, before all experiments, cells were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin solution, and grown in standard T75 tissue culture flasks at 37 °C. Since passage number was not constant for all experiments, it will be indicated in the appropriate section. For seeding, cells were lifted from the T75 flask surface by treating with 0.125% trypsin with EDTA for seven minutes and counted with a haemocytometer.
6.3 Initial cellular experiments

6.3.1 Growth of PAVICs on hydrogels

Initially, to qualitatively assess the spreading and morphology of PAVICs grown on gelMA, cells were seeded on dog-bone shaped hydrogels. Flat PDMS substrates were fabricated as described before (Chapter 4, Section 4.4.2) and 10 wt% gels (2% I2959 photoinitiator) were moulded inside the dog-bone shaped microwells as described before (Chapter 5, Section 5.2.2.1). The PDMS substrate was cut into smaller portions and each piece was placed inside the well of a six-well plate. Passage two cells were seeded on the hydrogels at 10,000 cells/cm². The cultures were monitored at day one and day three (Figure 6-1) and based on their appearance, it was concluded that the cells exhibited similar spreading and morphology to cells on glass. Therefore, this finding encouraged the use of the hydrogel system in the upcoming studies. Note that although the gel concentrations used here were not the same as the ones used in the cyclic experiments, it was expected that cells would attach, grow and proliferate comparably on the higher and slightly lower 20 wt% and 8.5 wt% gel concentrations, respectively [108].

![Day 1 (glass) - a), Day 1 (gelMA) - b), Day 3 (gelMA) - c)](image)

Figure 6-1 – PAVICs cultured on glass at day one (a), and on gelMA at day one (b), and day three (c) (images on gelMA only depict the straight section of the dog-bone hydrogel as that was the region of interest).

6.3.2 Time course α-SMA experiment

In order to assess the baseline level of α-SMA expression on gelMA, a time course experiment was conducted. This ensured that a minimal level of α-SMA was present before the cell-laden hydrogels were subjected to cyclic straining, thereby ensuring maximum dynamic range for induced α-SMA expression. All the hydrogels used in this study were 10 wt% (2% I2959 photoinitiator).
Initially, passage zero cells were directly seeded on dog-bone gels but exhibited initial low viability and poor spreading even after two to three days of culture. As such, it was decided that the cells would first be cultured on TCP, thereby selecting the healthy and adherent ones, followed by culture on the hydrogels.

6.3.2.1 Materials and methods

Round glass coverslips were first treated with 3-(trimethoxysilyl)propyl methacrylate (TMSPMA) (Sigma-Aldrich Canada Ltd.; Oakville, ON, Canada) to enhance the adhesion of gelMA to glass. The protocol was adapted from Nichol et al. [108]. GelMA was prepared as described previously (Chapter 5, Section 5.2.2) and the hydrogel-coated coverslips were placed in a 24-well plate. Thawed primary PAVICs were cultured in T75 flasks for four days prior to being seeded on the coverslips at a density of 20,000 cells/cm². For imaging, cells were immunostained for α-SMA and cell nuclei according to an established protocol (see Appendix D).

6.3.2.2 Results

It was found that 48 hours after seeding, the expression of α-SMA was acceptably low (Figure 6-2) and this time point served as the reference in the cyclic straining experiments.

Figure 6-2 - α-SMA expression in PAVICs on gelMA was minimal after 48 hr culture (arrows indicate α-SMA positive cells) (blue channel = nuclei; red channel = α-SMA).
6.4 Cyclic straining experiments

High-throughput proof-of-concept triplicate experiments were performed to measure the expression of α-SMA in PAVICs as a function of matrix stiffness, tensile straining, and chemical stimulation. As mentioned before, these stimuli are all important players in modulating the phenotype and cellular activity of PAVICs. The matrix stiffness condition comprised of two gelMA concentrations (8.5 wt% and 20 wt%) with a low and high stiffness (approximately 12 kPa and 43 kPa). These values were strategically chosen as it has been shown by Chen et al. [8] that the minimum required matrix stiffness to cause TGF-β1 induced myofibroblast differentiation was 22 kPa, with anything below this threshold exhibiting negligible α-SMA levels. The high and low moduli are also comparable to maximum moduli of the native fibrosa and ventricularis layers, respectively. In addition to modulating matrix stiffness in the presence of TGF-β1, we also included cyclic tension. In summary, modulating the matrix stiffness was achieved by varying the gelMA concentration, tensile tension was obtained via the staircase substrates and the actuation of the bioreactor arm, and chemical stimulus was achieved by adding TGF-β1. Therefore, investigating the combined effects of these parameters on myofibroblast differentiation of PAVICs first provided a perspective into their interactions and secondly, demonstrated the utility and high-throughput nature of the bioreactor system.

6.4.1 Experimental setup

Three experiments were run and each experiment consisted of four staircase substrates and four flat static controls. Each substrate represented a different condition. Two substrates were moulded with low stiffness gels (~12 kPa) and the remaining two with high stiffness gels (~43 kPa). The substrates were then seeded with PAVICs and incubated at 37 °C for 48 hours to ensure cell attachment and spreading on the gels. Immediately before stretching, one substrate from each pair was further treated with TGF-β1 at 5 ng/mL. The same was done to the static substrates. The staircase substrates were then cyclically stretched for 48 hours at a frequency of 1 Hz and amplitude of 7.0231 mm (in order to achieve the desired strains on the hydrogels).
6.4.2 Materials and methods

The procedures for fabricating the staircase and flat PDMS substrates as well as moulding the hydrogels into the microwells were identical to the ones described before (Chapter 4, Section 4.4 and Chapter 5, Section 5.2.2, respectively).

6.4.2.1 PAVIC seeding

To seed PAVICs onto the staircase substrates while keeping them horizontally leveled required the fabrication of a special container. To resolve this, a negative PDMS mould was cast around a staircase substrate. To fabricate the mould, an already fabricated staircase substrate was placed face down inside a deep polystyrene Petri dish (Nunc through Thermo Fisher Scientific; Rochester, NY, USA) and three 1” x 3” glass slides were placed in between the bottom of the dish and the staircase surface. This was done to make the mould deeper such that the substrate would sit lower than the mould surface thereby allowing cell media to be stored during cell culture. The Petri dish was placed in an 80 °C oven overnight. Once the PDMS cured, the substrate was taken out, leaving behind its impression. Any excess PDMS was trimmed manually.

All the equipment used was sterilized either via UV light or by spraying with 70% ethanol. Once the hydrogels were moulded into the staircase substrates, the substrates were placed in the PDMS moulds. The moulds themselves were placed in sterile deep Petri dishes and PBS with Ca²⁺/Mg²⁺ was poured over the hydrogels to keep them hydrated until PAVICs were seeded. The flat substrates were each placed into a well of a standard six-well plate and hydrated with PBS. PAVICs were seeded at 15,000 cells/cm² for the first experiment, and at 10,000 cells/cm² for the second and third. The density was lowered to reduce the cell-to-cell contact that may have triggered additional expression of α-SMA [143]. Once cells were seeded, the deep Petri dishes and the six-well plate were placed in an incubator at 37 °C for 48 hours.

6.4.2.2 Bioreactor assembly and clamping of substrates

Before the substrates were clamped inside the bioreactor, each component of the bioreactor was cleaned with detergent and autoclaved. The case and the base were too large to fit in the autoclave so they were thoroughly sprayed with 70% ethanol. The bioreactor was assembled inside the biological safety cabinet to maintain a sterile environment. The substrates were then
taken out of their PDMS moulds and gently placed inside a bioreactor well plate container, and clamped at one end. Once all four substrates were clamped at one end, they were then clamped at the other. This step was important in ensuring that all substrates experienced an identical and synchronous strain during stretch. The flat substrates were taken out of the six-well plate and placed in smaller individual Petri dishes. The dishes were placed in the bioreactor with their lids off.

6.4.2.3 Substrate imaging

Once the experiment concluded, the substrates were fixed and immunostained for α-SMA, F-actin and cell nuclei according to an established protocol (see Appendix D). To obtain high quality images, a portion of the bottom of a polystyrene Petri dish was trimmed and replaced with a glass cover slip. The substrates were then cut into pieces along the substrates step edges and placed on the coverslip. A few drops of PBS were deposited on the coverslip to keep the environment humid. Imaging was performed with a fluorescent microscope (Model IX71, Olympus; Center Valley, PA, USA) connected to a camera (Model Retiga 2000R, Qimaging; Surrey, BC, Canada).

6.4.2.4 α-SMA counting

To consistently count α-SMA positive cells on all images, a standard that defined which cells were positive and which were negative was created (Figure 6-3).

Figure 6-3 – Representative images indicating negative expression of α-SMA (a), and positive expression (b) (yellow arrows point to cells that although fluoresce, they do not exhibit fibers; white arrows point to α-SMA positive cells; yellow lines delineate the gel edges) (blue channel = nuclei; red channel = α-SMA).
Cells that appeared to exhibit a fibrillar nature were counted as positive and cells that did not were counted as negative. Furthermore, cells that were mildly expressing α-SMA fibers were also counted, as they may have indicated the presence of proto-myofibroblasts [51].

### 6.4.3 Statistical analysis

To evaluate the α-SMA expression as a function of matrix stiffness, chemical stimulant, and mechanical strain level, three-way ANOVA and post-hoc Student-Newman-Keuls pairwise comparisons were performed (n = 3 experiments in all cases). To further evaluate the data, students t-tests as well as one-way ANOVA analyses coupled with post-hoc Student-Newman-Keuls pairwise comparisons were performed on the matrix and chemical conditions for each nominal strain level, as explained in the section below. In the case of equal variance failing, one-way ANOVA on ranks and Mann-Whitney Rank Sum tests were run. The α-SMA levels across the seven dog-bone hydrogels on every staircase substrate step were averaged. Therefore, each substrate step contained a single value for α-SMA expression, corresponding to the appropriate nominal strain region. All data are reported as mean ± standard deviation with p ≤ 0.05 considered statistically significant.

### 6.4.4 Results and discussion

The dog-bone hydrogels on the 15% (1.33 mm thickness) and 20% (1 mm thickness) nominal strain conditions were lost in all cases. This was attributed to the ears of the hydrogels either slipping over the PDMS posts or failing altogether, and this was confirmed during imaging.

It was found that the high stiffness gels were statistically different from the low stiffness gels (p < 0.001) and the TGF-β1 conditions were statistically different from the no-TGF-β1 conditions (p < 0.001). In addition, the static condition as well as the 10% (2 mm thickness) and 5% (4 mm thickness) nominal strain conditions were all statistically different from the 2.5% (8 mm thickness) nominal strain condition (p < 0.05) (Figure 6-4). However, there were no statistical differences between the static, 5% and 10% nominal strain conditions.

To further investigate the source of the statistical differences, one-way ANOVA tests were run between the nominal strain levels on each of the four conditions. The tests revealed that α-SMA expression was not statistically different between strain levels on any of the four conditions, with
the exception of one. On the 8.5 wt% without TGF-β1 condition, α-SMA expression on the static, 10% and 5% nominal strain levels were statistically higher (p < 0.05) than on the 2.5% strain level and the mean expression was greater by 110%, 200% and 170%, respectively (Figure 6-5). This suggested that the presence of TGF-β1 as well as an increase in matrix stiffness can potentially wash out the effects realized by mechanical straining alone, which has been shown to induce myofibroblast differentiation [61]. Matrix stiffness and TGF-β1 are both well-known inducers of myofibroblast differentiation [50, 52] and they could potentially act in concert with mechanical tension to raise α-SMA expression. One interpretation is the possible presence of a

Figure 6-4 – Results comparing mean α-SMA expression between gel stiffness, chemical stimulus (TGF-β1) and nominal strain level (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). Three-way ANOVA revealed that the α-SMA expression on static, 10% and 15% nominal strain levels were statistically different from 2.5% strain levels. Results are presented as mean ± standard deviation (* p < 0.05 and n = 3).
marked threshold (5% nominal strain in this case) that causes fibroblasts to undergo myofibrogenesis on soft gels and in the absence of TGF-β1. The high expression on the static controls could be associated with a higher cell-to-cell contact, which was predominantly greater on the static controls, that has been shown to induce myofibrogenesis [143]. Alternatively, Galie et al. [144] showed decreased α-SMA expression in cardiac fibroblasts encapsulated in collagen gels under the presence of 5% strain compared to static controls after 48 hours, although the study was done in 3D and may not be applicable here. Further analyses are still required.

![Graph](image)

**Figure 6-5** – One-way ANOVA results comparing mean α-SMA expression on 8.5 wt% soft gels without the influence of TGF-β1 across all nominal strain levels (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). Static, 10% and 5% nominal strain levels were all significantly different from the 2.5% nominal strain level. Results are presented as data ± standard deviation (*p < 0.05 and n = 3).

Student t-tests further showed that TGF-β1 did not induce a statistically higher α-SMA expression on soft gels (Figure 6-6) with p = 0.7, 0.653 and 0.068 on the 5%, 10% nominal strain levels and the static condition. The 2.5% nominal strain level was statistically significant with p = 0.003. However, TGF-β1 induced statistically higher levels of α-SMA on the stiffer gels across all nominal strain levels (Figure 6-7) with p = 0.009, 0.05, 0.018 and < 0.001 and means were
greater by 101%, 90%, 87% and 185% on the 2.5%, 5%, 10% nominal strain levels and the static condition, respectively. This result excellently corroborated the findings reported by Chen et al [8], who demonstrated a marked decrease in TGF-β1 induced myofibroblast differentiation on gels with stiffnesses lower than 22 kPa. The mechanism by which TGF-β1 is secreted and recruited by cells has been proposed before [11]. Endogenously secreted TGF-β1 is stored in a latency-associated protein (LAP) complex that is directly linked to the cell cytoskeleton via integrins and is anchored to the ECM. On soft, compliant substrates, cell-generated tension acts through the LAP complex and deforms the substrate. The TGF-β1 thus remains sequestered and cannot bind to its receptors, causing the cells to degrade α-SMA and generate progressively less and less new α-SMA, losing its contractile phenotype. However,

Figure 6-6 – Results comparing mean α-SMA expression on 8.5 wt% soft gels with and without the influence of TGF-β1 across all nominal strain levels (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). TGF-β1 induced a statistically higher α-SMA expression on the 2.5% nominal strain level. Results are presented as data ± standard deviation (**p < 0.005 and n = 3).
when the substrate is stiffer (E >> 10 kPa), the cell-generated tension is resisted by the rigidity of the matrix and causes the LAP complex to open, thereby releasing soluble TGF-β1. This positive loop then causes the synthesis of more LAP complexes, ECM and α-SMA, and maintains the myofibroblast phenotype. This mechanism can likely explain the differences between TGF-β1 induced myofibroblast differentiation on our soft and stiff gels. Backtracking, the effect of external mechanical tension may facilitate the release of TGF-β1 by causing more strain on the LAP complex and may explain why, at higher mechanical strain levels, and without the influence of exogenous TGF-β1 or high matrix stiffness, α-SMA levels tend to increase. Of course, in our experiments, earlier results are somewhat confounded by the high levels of α-SMA on the static

Figure 6-7 – Results comparing mean α-SMA expression on 20 wt% stiff gels with and without the influence of TGF-β1 across all nominal strain levels (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). TGF-β1 induced a statistically higher α-SMA expression across all nominal strain levels and on the static control. Results are presented as data ± standard deviation (***p < 0.001, *p < 0.05, and n = 3).
controls. The aforementioned confounding result (i.e., statistically higher $\alpha$-SMA levels only on the 2.5% nominal strain level on soft gels) may suggest that higher strain levels result in mechanically induced $\alpha$-SMA to high enough levels that the addition of TGF-$\beta1$ does not cause a significant increase, whereas the mechanical contribution at the 2.5% nominal strain level is low and there is potential for TGF-$\beta1$ to induce myofibrogenesis.

Furthermore, matrix stiffness with TGF-$\beta1$ induced statistically higher $\alpha$-SMA expression on the 2.5% and 10% strain levels, and on the static substrates with $p < 0.001$, $= 0.016$ and 0.01, and the $\alpha$-SMA mean levels were greater by 200%, 125%, and 106%, respectively (Figure 6-8).

![Figure 6-8](image.png)

**Figure 6-8** – Results comparing mean $\alpha$-SMA expression on TGF-$\beta1$ treated soft and stiff substrates across all nominal strain levels (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). In the presence of TGF-$\beta1$, matrix stiffness induced statistically higher $\alpha$-SMA expression across the 2.5% and 5% nominal strain levels, and on the static control. Results are presented as data ± standard deviation ($**p < 0.001$, $^*p < 0.05$, and $n = 3$).
Matrix stiffness without TGF-β1 induced statistically higher α-SMA expression on the lowest nominal strain level (2.5%) and on the static substrates with \( p = 0.026 \) and 0.006, and the α-SMA mean expression levels were greater by 207% and 58%, respectively (Figure 6-9). Interestingly, on the conditions without TGF-β1, the effect of matrix stiffness is only apparent on the control and the lowest nominal strain level. This suggests that at low or zero strains, soft gels are not capable of inducing α-SMA expression alone. Stiffer gels, on the other hand, are most likely experiencing a marked increase in α-SMA levels via autocrine secretion of the LAP complex, which releases soluble TGF-β1 and drives the process forward. However, the addition of external

![Figure 6-9](image)

**Figure 6-9 –** Results comparing mean α-SMA expression on soft and stiff substrates in the absence of TGF-β1 across all nominal strain levels (8 mm, 4 mm, and 2 mm substrate thicknesses correspond to 2.5%, 5%, and 10% nominal strain levels, respectively). In the absence of TGF-β1, matrix stiffness induced statistically higher α-SMA expression across the 2.5% nominal strain level and on the static control. Results are presented as data ± standard deviation (*\( p < 0.05 \) and \( n = 3 \)).
tension increases the \( \alpha \)-SMA levels of the softer gels and it may be that the LAP complex experiences extra “pull” from the matrix and the cell and releases TGF-\( \beta \)1 reserves. The presence of exogenous TGF-\( \beta \)1 seems to increase \( \alpha \)-SMA expression even further, almost regardless of external tension magnitude. It becomes clear that the effects of matrix stiffness and presence of TGF-\( \beta \)1 are evidently coupled, if not synergistic, and this is a finding supported by the literature [11, 51, 52]. To complicate matters, higher stiffness gels fabricated at the same degree of methacrylation experience intrinsically more cell-binding ligands than lower stiffness gels since there is more gelatin by weight. Therefore, increased cell adhesion on the higher stiffness gels may have been, at least in part, an artifact of the chemistry of the hydrogels. This could have also contributed to an increase in \( \alpha \)-SMA expression.

6.5 Conclusion

In summary, microfabricated substrates containing cell-laden hydrogels were clamped in a bioreactor system and were screened combinatorially for the effects of matrix stiffness, mechanical tension, and influence of chemical stimuli on the expression of \( \alpha \)-SMA, a marker that indicated the transition of quiescent fibroblasts into contractile and disease-associated myofibroblasts. Analysis of the results showed that both matrix stiffness and TGF-\( \beta \)1 significantly increased \( \alpha \)-SMA levels (\( p < 0.001 \)), while the effect of mechanical strain was only significant on soft gels (~12 kPa) without TGF-\( \beta \)1. Further analyses revealed the potentiating effects of matrix stiffness and TGF-\( \beta \)1 on \( \alpha \)-SMA expression and showed their key importance in regulating cellular events that initiate myofibrogenesis.
Chapter 7

7 Conclusion and future recommendations

7.1 Conclusions

The first main objective of the thesis was to conceptually design and manufacture a bioreactor system for high-throughput multifactorial screening of cell-seeded constructs or microtissues that could apply mechanical tension, modulate the matrix stiffness, and introduce biochemical stimuli. The second main objective was to run factorial experiments and investigate the independent and combined effects of the aforementioned stimuli on the expression of pathological markers, more specifically α-SMA, in PAVICs.

To that extent, first, a bioreactor vessel was conceptually designed and manufactured. The vessel contained eight compartments with a set of clamps in each compartment and this enabled the clamping of substrates with various geometries. The clamps were connected to transversal static bars, which were bolted down, and moving bars, which were welded to a main actuating arm. The main actuating arm was connected to an actuating rod that interfaced with a stepper motor through a rack and pinion mechanism. The stepper motor could be programmed as desired and transmitted linear cyclical motion to the actuating arm. It was found that the input displacement to the stepper motor correlated well with output displacement inside the bioreactor. Furthermore, it was shown that the bioreactor supported cell cultures comparably well to a standard incubator and maintained aseptic conditions for up to one week.

To address the need for high-throughput, a flexible substrate microfabricated from PDMS that could be clamped inside the bioreactor vessel was designed. The substrate featured a staircase geometry and contained five regions along its length that exhibited different strain profiles during stretch. The substrate was further micropatterned with an array of 35 dog-bone shaped microwells (seven per strain region x five regions). The microwells were designed to enable cell cultures or tissue maintenance (and retention) and provide uniform longitudinal strain to the growing constructs. FEA analyses were conducted to predict engineering substrate strains at the microwell locations and these data were validated with empirically collected microwell strains. It was also shown that the microwell strain values featured low variability between substrates.
A photocrosslinkable hydrogel system, gelatin methacrylate, was incorporated and characterized
within the substrate. Gelatin methacrylate was chosen due its excellent micropatterning
properties, easily tunable stiffness, and support of adhesion, proliferation and organization of cell
cultures. A protocol was developed to mould the hydrogels into the dog-bone microwells, and it
was shown that engineering longitudinal hydrogel strains exhibited similar values to substrate
strains at the same locations. Some discrepancies were nonetheless observed and this was
attributed to the loss of hydrogel adhesion from the substrate underneath. Further, the hydrogels
displayed uniform strain profiles in their straight regions, a result that was desirable and
validated solid mechanics dog-bone theory.

The bioreactor vessel, staircase substrate, and hydrogel system were ultimately integrated and
used in proof-of-experiment multifactorial investigations on the effects of mechanical strain,
matrix stiffness and biochemical stimuli on α-SMA expression in PAVICs. Analysis of the
results showed that both matrix stiffness and biochemical stimulus (TGF-β1) significantly
increased α-SMA levels (p < 0.001), while the effect of mechanical tension was only significant
on soft gels (~12 kPa) without TGF-β1 (p < 0.05).

7.2 Future recommendations

A large portion of the study was dedicated to ensuring the microwell moulded dog-bone
hydrogels did not detach or float away from their locations. Although effects of lower tensile
strains were investigated, hydrogels residing on the higher strain regions (15% and 20% nominal
strain regions) were unusable because they slipped out of their moulds and thus did not
experience the appropriate strain. A more efficient trapping mechanism is required, perhaps
larger overhangs, and this might involve increasing the dimensions of the dog-bones. Because
this would decrease the number of technical replicates, a change in substrate geometry may also
be required. Further, it is recommended that the regions of high strain be well away (>10 mm)
from the clamps to avoid any clamping and handling induced disturbances. In addition, the
substrate clamping procedure requires streamlining as it always involved extensively
manipulating the hydrogel moulded substrate. This might have also caused unwanted
disturbances to the gels.

A more robust way of attaching the stepper motor mounting bracket to the bioreactor baseplate is
required. Since the teeth on the motor shaft gear were not at the same level as teeth on the rack,
makeshift spacers from PTFE were created. However, due to the flexibility of PTFE, over
tightening or under tightening the motor mounting bracket bolts resulted in either too much
downward force being applied to the rack by the gear, or the teeth not meshing well,
respectively. For over tightening, this would result in excessive friction between the gear and
rack teeth as well as between the moving transversal bars and the side rails inside the bioreactor.
The motor would have to work extra hard and material would be slowly shaved off the side rails.
Under tightening could result in badly damaging the gear and rack teeth, as they would collide
during each cycle due to improper meshing. A sweet spot was achieved by varying the number
of spacers and constantly monitoring the amount of tightening. However, this was very time
consuming.

For the biological studies, several changes could be implemented to better mimic physiological
environments or increase the combinatorial nature of the experiments. Firstly, future studies
should aim to stretch hydrogel constructs that contain encapsulated cells. It has been shown that
signaling and cell events differ in two- versus three-dimensional environments [145]. Three-
dimensional studies are considered more physiologically relevant and might elucidate further
effects of stimuli that cannot be replicated in two-dimensional systems. Furthermore, to mimic
the cyclic loading pattern sustained by the heart valve in vivo, PAVICs could be subjected to
more physiological loading patterns. Highly combinatorial experiments can be achieved by
including a larger number of matrix stiffness conditions, stimulating with more biochemicals, or
incorporating different types of ECM. The experiments in this study used four of the eight
compartments and future experimental setups could be factorially designed to take advantage of
the remaining space.
References


Appendices

Appendix A – Stepper motor torque calculations

Calculations were performed to determine the amount of torque required to actuate the maximum number of substrates (eight) and overcome inertial loads due to acceleration of the actuating arm.

Given:

Pinion pitch radius (r): 5.25 mm
Required linear displacement (d): 7.0231 mm
PDMS Young’s modulus (E): 2 MPa
Substrate width (w): 27.5 mm
Substrate thickness (t) (on the 20% nominal strain (ε) step): 1 mm
Actuating arm weight: 0.7 kg
Pinion angular acceleration (θ): 5 rev/s²

Total force required (stretch eight substrates and actuate the actuating arm)

First derive an equation that incorporates all the given information above to calculate force required to stretch one substrate by the required linear displacement. Simplify all staircase cross-sections to a single cross-section (1 mm) that runs alone the whole length of the substrate and assume that it stretches by 20%. Therefore:

Since \( \sigma = E \ast \varepsilon \) and \( \sigma = \frac{F}{A} \), we obtain that \( F = A \ast \sigma = A \ast E \ast \varepsilon = w \ast t \ast E \ast \varepsilon \)

Therefore, the force required to stretch one substrate is:

\[
F = 0.0275 \text{ m} \ast 0.001 \text{ m} \ast 2 \ast 10^6 \text{ Pa} \ast 0.2 = 11 \text{ N}
\]

The force required to stretch eight substrates in parallel is thus 88 N.

To calculate the force to actuate the actuating arm, we know that \( F = m \ast a \) but we do not know the linear acceleration (\( a_L \)). However, we know the angular acceleration is 5 rev/s² = 31.415 rad/s²; we also know that the pitch radius of the pinion is 5.25 mm. Therefore:

\[
a_L = \theta \ast r = 31.415 \frac{\text{rad}}{s^2} \ast 0.00525 \text{ m} = 0.165 \frac{m}{s^2}
\]

Therefore, the force required to actuate the actuating arm is

\[
F = m \ast a = 0.75 \text{ kg} \ast 0.165 \frac{m}{s^2} = 0.124 \text{ N}
\]

The total force required to stretch eight substrates and actuate the actuating arm is thus 88.124 N.
Required torque

We know that $\tau = F \times r$ where $F$ is the total force and $r$ is the pinion pitch radius.

Therefore $\tau = F \times r = 88.124 N \times 0.00525 m = 0.463 Nm$

The stepper motor is therefore required to produce at least twice as much torque (since it is run at half of the rated current). The motor we selected is the HT23-399 stepper motor with a holding torque of 1.864 Nm. The holding torque is four times greater than the required torque which means that even at half the current (therefore half the torque), the motor still has twice the torque required. Thus, this was deemed acceptable.
Appendix B – Bioreactor system programming

This protocol outlines the steps taken to interface the step motor driver, which controlled the stepper motor, with a personal computer. This enabled the programming of different loading profiles into the driver that were transmitted to the stepper motor. The protocol focuses on the loading profile used in this study.

Step motor driver interfacing

1. Connect the supplied step motor driver cable (Model RS-232 PC/MMI, Applied Motion; Watsonville, CA, USA) to a USB-to-serial DB39 adaptor cable (CablesToGo; Moraine, OH, USA) and install the appropriate driver (drivers found at: https://www.cablestogo.com/help/tech_support.asp# under “Drivers and manuals” link).

2. **IMPORTANT:** Before proceeding further, ensure that the motor leads are connected to the step motor driver and that the USB-to-serial DB39 adaptor cable is connected to the RS-232 PC/MMI cable.


4. Install and open SiProgrammer™ (a prompt may appear saying that a communication error has occurred. Click “OK” until it disappears).

5. Connect the USB-to-serial DB39 adaptor cable to a USB port on the PC.

6. Supply power to the step motor driver.

7. Select the appropriate COM port corresponding to the USB port and ensure that the right motor driver version is displayed (Si2035 in this case).

Once power is supplied to the step motor driver, it will automatically start running the last program that was downloaded into it. To stop the motor, click “Upload”. The program consists of three lines: the first rotates the pinion and displaces the rack by 7.0231 mm (displacement required to achieve the needed substrate strains); the second line rotates the pinion in the opposite direction and retracts the rack, and the third line is a loop that repeats the process. The linear speed of the rack is such that it oscillates at 1 Hz.

Step motor driver programming

1. To enter the first line of the program, click the downward arrow beside the number 1.

2. A menu appears that outlines a number of programming options. Click “Feed to Length” to open a submenu that allows for the control of rotation speed, acceleration, etc.

3. Convert the desired linear displacement (7.0231 mm) into angular displacement based on the number of steps by which the motor needs to rotate. Knowing the motor has 20,000 steps/rev
and the diameter of the pinion, calculate the required number of steps (4258 steps in this case). Input the number in the “Distance” field.

4. Convert the desired linear speed into angular speed using a similar methodology to achieve a frequency of 1 Hz. Input the number (0.4258 rev/s in this case) in the “Speed” field.

5. Lower the acceleration and deceleration to 5 rev/s² to reduce the incurrence of high inertial loads.

6. Set the direction to CCW in the “Direction” option. This ensured that the pinion actuated to stretch, not compress, the substrates.

7. To enter the second programming line, click the downward arrow beside the number 2.

8. Repeat steps 2-5. Ensure the direction is set to CW.

9. To enter the third programming line, click the downward arrow beside the number 3.

10. Select the programming option “Go To”.

11. A menu appears that prompts the user to enter the number of a programming line. Enter number 1. This causes the program to loop between the first two programming lines.

12. Turn the “Idle Current” option to 100% in the main menu and change the current value to 0.7 A/Phase.

13. Click “Download” to download the program into the step motor driver.

14. To test the program, click “Execute”.

To disconnect the motor, perform the aforementioned connecting sequence backwards. To run the motor during an experiment, ensure the appropriate program is downloaded into the step motor driver, and power up the driver, which will turn on the motor. Connection to a PC is not required during biological experiments.
Appendix C – SU-8 master fabrication

The SU-8 masters were all fabricated in the Emerging Communications Technology Institute (ECTI) cleanroom facility.

Ensure an ample supply of cleanroom wipes is available and is placed on the wet bench area where the work will be performed. This is to quickly clean up accidental spills of chemicals. Use digital hotplates and ensure they are level. Use cleanroom wipes as spacers, if needed. Before starting, it is highly recommended that the glass slides be cleaned with Piranha solution (separate procedure), to clean the glass surface of any organic residues.

Seed layer fabrication (~7 μm)

1. Rinse slides with acetone and IPA, and blow dry with nitrogen.
2. Perform a dehydration bake by heating the slides to 180 °C for 20 min and allow the slides to cool with the hotplate to ~60 °C.
3. Using a plastic pipette, drop a few mL of SU-8 5 onto the surface of the slides. Angle the slides to coat them fully.
4. Spin coat the slides using the following protocol:
   a. Step 1: 5 s, 500 RPM, 88 ACL
   b. Step 2: 30 s, 500 RPM, 88 ACL
   c. Step 3: 15 s, 3000 RPM, 528 ACL
   d. Step 4: 30 s, 3000 RPM, 528 ACL
5. Prebake the slides at 65 °C for 2 min and at 95 °C for 5 min, respectively.
6. Allow the slides to cool with the hotplate to ~60 °C.
7. Using a photomask, expose the slides with UV light for 2 s by selecting the FLOOD-6 option on the mask aligner.
8. Post-bake the slides at 65 °C for 1 min and at 95 °C for 4 min, respectively.
9. Allow the slides to cool with the hotplate to ~60 °C.
10. Develop the slides in SU-8 developer for 2 min.
11. Rinse with IPA (NOT acetone) and blow dry with nitrogen. Ensure that no white deposits are present on the surface. If this is the case, place the slides in developer for an additional 5 min.
12. Hard-bake at 180 °C for 30 min.

Feature fabrication (120 μm or 300 μm, depending on number of spins)

1. Rinse seed-layer slides with IPA (NOT acetone), and blow dry with nitrogen.
2. Perform a dehydration bake by heating the slides to 180 °C for 20 min and allow the slides to cool with the hotplate to ~60 °C.

3. Pour enough SU-8 50 (either from the bottle or from an aliquot) onto the surface of the slides to partially coat it. Burst any bubbles with a 20-gauge needle, as needed.

4. Spin coat the slides using the following protocol:
   a. Step 1: 5 s, 500 RPM, 88 ACL
   b. Step 2: 30 s, 500 RPM, 88 ACL
   c. Step 3: 5 s, 1000 RPM, 352 ACL
   d. Step 4: 33 s, 1000, 352 ACL

5. Prebake the slides at 65 °C for 10 min and at 95 °C for 30 min, respectively.

6. Allow the slides to cool with the hotplate to ~60 °C.

7. For slides with 120 μm features, go to the next step. If creating 300 μm features, repeat step 4 and prebake the slides at 65 °C for 10 min and at 95 °C for 45 min, respectively. Allow the slides to cool with the hotplate to ~60 °C.

8. Using a photomask, expose the slides with UV light for 30 s (120 μm), or 45 s (300 μm) by selecting the SOFT-30 option on the mask aligner.

9. Post-bake the slides at 65 °C for 2 min (120 μm) or 3 min (300 μm), and at 95 °C for 15 min (120 μm) or 22 min (300 μm), respectively.

10. Develop the slides in SU-8 developer for ~20 min (120 μm) or 40 min (300 μm). If developing for 40 min, replace the developing solution after 20 min.

11. Rinse with IPA (NOT acetone) and blow dry with nitrogen. Ensure that no white deposits are present on the surface. If this is the case, place the slides in developer for an additional 5 min.

12. Hard-bake at 180 °C for 1 hr (120 μm), or at 80 °C for 3 days (300 μm).
Appendix D – Direct immunostaining protocol

Reagents needed:

1. PBS with Ca^{2+}/Mg^{2+} for rinsing
2. 10% Neutral Buffered Formalin (NBF)
3. 0.1% Triton X-100
4. Monoclonal Anti-Actin Muscle-Cy3 antibody produced in mouse (Cat #: C6198, Sigma-Aldrich)
5. Phalloidin, fluorescein isothiocyanate labeled (Cat #: P5282, Sigma-Aldrich)
6. 3% Bovine Serum Albumin (BSA) in PBS
7. 1 μg/mL Hoechst nuclear stain

Procedure

1. Fixation and permeabilization
   a. Wash cells twice with PBS for 5 min each.
   b. Fix 5-10 min in 4% PFA or 10% NBF at room temperature.
   c. Remove fixative and rinse twice with PBS for 5 min each.
   d. Permeabilize with 0.1% Triton X-100 for 5 min.
   e. Rinse twice with PBS for 5 min each.
   f. Fixed and permeabilized cells can be stored in PBS at 4 °C, if necessary.

2. Staining
   a. Block with 3% BSA for 20 min at 37 °C.
   b. Dilute 1:100 phalloidin and anti-actin muscle antibodies in 3% BSA.
   c. Incubate at room temperature for 1 hr.
   d. Rinse with PBS for 5 min.
   e. Add Hoechst diluted 1:1000 in PBS for 5 min to stain nuclei.
   f. Rinse twice with PBS for 15 min each.
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