Engineering Cardiac Valve Extracellular Matrix Structure and Function for Modeling Pathogenesis and Regeneration

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Engineering Cardiac Valve Extracellular Matrix Structure and Function for Modeling Pathogenesis and Regeneration

A dissertation presented

by

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to

John A. Paulson School of Engineering and Applied Sciences

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Fibrosis of the semilunar cardiac valves clinically manifests as deterioration in late-stage functional performance. Stenotic insufficiencies, due to either congenital or acquired heart valve disease, are a result of irreversible structural aberrations to the normally highly ordered leaflet extracellular matrix (ECM). In particular, excessive remolding and deposition of ECM in the load bearing fibrosa layer of the leaflets over time leads to tissue thickening and eventual calcification. Therefore, we asked what role the fibrosa layer, essential for healthy leaflet mechanical functionality, plays in early-stage valve disease etiology and function. We hypothesized that recapitulating the valvular fibrosa ECM structure and composition will enable the self-assembly of valvular tissue to model acute fibrotic pathogenesis and provide a platform for functional tissue replacement. We tested this hypothesis by engineering both in vitro and in vivo models of semilunar valve tissues. In vitro, aortic valve interstitial cell (AVIC) tissues were engineered on two dimensional, flexible thin films and fibrous scaffolds to mimic fibrosal alignment. Engineered AVIC tissues were acutely exposed to a clinically relevant dose of the drug Pergolide, a known valvulopathogen, in order to determine early, drug-induced changes in fibrotic biomechanical function. AVIC tissues on thin films lost nearly half of their capacity to generate both basal and active tissue tone, indicative of a switch from a reparative, contractile cell phenotype to a more motile and synthetic one as a result of treatment with the drug. These data were supported by a decrease in both tissue alignment and expression of the contractile protein alpha smooth muscle actin along with increased coflin-actin
colocalization in drug-treated tissues. Additionally, AVIC tissues engineered on two-dimensional, fibrous scaffolds showed slightly increased biaxial stiffness when treated acutely with the drug, suggestive of the drastic increases in stiffness observed clinically in cases of valve fibrosis. We then utilized our experience building two dimensional valve tissue models in vitro to engineer three dimensional fibrous scaffolds designed to both function immediately upon implantation as well as provide a platform for tissue regeneration in vivo. We developed an automated jet-spinning collection process to manufacture seamless, three dimensional semilunar valve scaffolds, maintaining the fibrosal alignment within leaflets. A novel, two-piece mandrel collection system enabled rapid, scalable, and controlled production of these semilunar “JetValve” scaffolds. We tailored JetValve fiber size, alignment, biaxial stiffness, and composition to recapitulate that of the native pulmonary valve leaflet fibrosa (ovine model). JetValves were functionally tested using an in vitro pulse duplicator system as well as implanted using minimally invasive techniques into the pulmonary valve position in an adult, ovine model for 15 hr. Both in vitro and in vivo tests showed JetValve performance similar to native with both a small closing volume during diastole and minimal pressure gradient across the leaflets during systole. Initial histological examination showed intact, explanted JetValves with non/minimal clotting and full-thickness neutrophil infiltration at 15 hr. Initial explants at one month time points reveal endothelialization of the leaflet surface and early cellular penetration into the scaffold. Taken together, these data demonstrate that engineered semilunar heart valve fibrosa of can serve as both a basis for both early-stage valvular tissue disease modeling as well as the functional basis of scaffolds designed for tissue replacement.
# Table of Contents

Abstract ........................................................................................................................................ iii

Table of Contents .......................................................................................................................... v

Table of Figures ............................................................................................................................. ix

Acknowledgements ........................................................................................................................ xii

1  Introduction ................................................................................................................................ 1

  1.1  Engineering Models for Drug Testing with Organs on Chips ............................................. 3

    1.1.1  Pharmaceutical Demand: Modeling the Patient *In vitro* ......................................... 3

    1.1.2  Design: Creating the Spatiotemporal Blueprints of Organs on Chips ....................... 5

    1.1.3  Build: Choosing the Materials and Tools for Building Organs on Chips ............... 8

    1.1.4  Test: Correlating Organs on Chips to the Patient ...................................................... 10

    1.1.5  Conclusion for Engineering Organs on Chips ........................................................... 13

  1.2  Fibrous Scaffolds for Engineering Regenerative Cardiac Scaffolds ............................... 14

    1.2.1  Fiber-Based Scaffolds for Regenerative Tissue Engineering .................................. 15

    1.2.2  Design Criteria for Engineered Cardiac Valve Tissues .......................................... 17

    1.2.3  Fibrous Scaffold Production Techniques ................................................................. 23

    1.2.4  Cardiac Valve Tissue Repair Using Fibrous Scaffolds ........................................... 31

    1.2.5  Conclusions for Engineering Fibrous Scaffolds ....................................................... 34

  1.3  Graphical Approach ............................................................................................................... 35

2  *In vitro* Modeling of Drug-Induced Fibrosis in Valvular Interstitial Cell Tissues ........ 36

  2.1  Introduction ......................................................................................................................... 37

  2.2  Materials and Methods ....................................................................................................... 39

    2.2.1  Aortic Valve Interstitial Cell Source and Isolation .................................................... 40

    2.2.2  Engineered Aortic Valve Interstitial Cell Tissues ..................................................... 42

    2.2.3  Aortic Valve Interstitial Cell Tissue Tone Stress Assay ........................................... 43

    2.2.4  Immunostaining and Tissue Alignment Analysis ....................................................... 47

    2.2.5  Western Blot Protein Expression Analysis .................................................................... 48
3.4 Discussion ............................................................................................................. 85
3.5 Conclusion............................................................................................................. 89

4 Conclusions.............................................................................................................. 90
  4.1 Overarching Themes and Strategy ........................................................................ 90
  4.2 Engineered Microphysiological Tissues for Modeling Disease ......................... 90
  4.3 Manufacturing Biomaterials for Regenerative Tissue Engineering .................... 93
  4.4 Future Research Directions ................................................................................ 95
  4.5 Future Challenges for Engineering Cardiovascular Tissues ............................... 97
  4.6 Funding Sources ................................................................................................ 102

5 Bibliography ........................................................................................................... 104

6 List of Publications and Patents ............................................................................. 135
  6.1 Appendix A: List of Publications ........................................................................ 135
  6.2 Appendix B: List of Patents ................................................................................ 136
# Table of Figures

Figure 1.1: The Design, Build, and Test algorithm for a Brain-on-Chip................................. 5

Figure 1.2: Clinical Function Diagnostics versus Typical Organ Chip Readouts. ............ 13

Figure 1.3: Cellular/Tissue Mechanotransduction through the Fibrous ECM.................. 15

Figure 1.4: Structure of the Aortic Valve in Health and Disease. ................................. 22

Figure 1.5: Strategies for Building Fibrous Scaffolds. ................................................. 24

Figure 1.6: Overview of Bio-Inspired Cardiac Fibrous Scaffold Fabrication. .................. 32

Figure 1.7: Approach to Improving Heart Valve Disease Diagnosis and Repair. .......... 35

Figure 2.1: Recapitulating the Leaflet Fibrosa with Biohybrid Rotary Jet Spinning ...... 41

Figure 2.2: Fibrosa Contractile Thin Film Assay Fabrication. ....................................... 45

Figure 2.3: Pergolide Induced Tissue Stiffening on Fibrous Scaffolds......................... 51

Figure 2.4: Pergolide Induced Tissue Stiffening on Fibrous Scaffolds......................... 52

Figure 2.5: Pergolide Induced Loss of Tissue Tone Generation and Anisotropy......... 54

Figure 3.1: Automated Rotary Jet Spinning of JetValve Manufacturing ......................... 64

Figure 3.2: Mandrel Scaling and Aortic Sinus Inclusion.................................................. 66

Figure 3.3: JetValve Catheter-Based Deployment and Crimping................................. 73

Figure 3.4: Biohybrid Structural and Stiffness Characterization................................. 77

Figure 3.5: Surface Protein Content of RJS and Electrospun Fibers............................. 78

Figure 3.6: Biohybrid Crystallinity and Prepared Shelf Life......................................... 80

Figure 3.7: Biaxial Stiffness at 1 Week Hydration......................................................... 81

Figure 3.8: Batch Process Capability Quality Control.................................................... 82

Figure 3.9: *In Vitro* and *In Vivo* Functional Performance........................................ 84
Table of Tables

Table 1.1: Structural and Functional Properties of Cardiac Valves.............................. 20
Table 1.2: Fibrous Scaffold Fabrication Techniques......................................................... 25
Acknowledgments

Early in my graduate studies I was told that a PhD is a ‘state of mind’. Hearing that was as comforting as studying solid mechanics and fluid dynamics using just Greek symbols like I did in my first semester courses here at Harvard — not very. That was a difficult semester; graduate school would not be as straightforward as checking off classes and learning some new techniques in the laboratory, it has not been just a matter of putting in the time. Early on it became clear that to achieve that PhD ‘state of mind’ would require significant courage and perseverance; I would not have been able to do it without my family.

I want to first thank my wife, Valerie, who has been by my side since we were freshmen in high school, always supportive of what I need and want to do. There are lots of highs and lows in graduate school and sometimes those can follow you home when they shouldn’t; I want to thank Val for putting up with me and making sure that I kept my head on straight in order to make it through this. My parents, Keith and Louise, in addition to my brothers, Jim and Domenic, have also always been full of encouragement and advice as well as a crowd that I could vent to in our own northeast dialect — let’s call it — when I needed. You won’t catch me saying this too often and I’m cringing writing it down now, but I want to thank my family for their persistent Harvard-centric banter over the years — it’s helped me keep everything in perspective: work shouldn’t be taken too seriously, there is much more to life. And finally, there’s Gabe, my boy. I’m not too sure I really understood exactly why I was doing all of this until Gabe came along. Doing my part to advance science… to someday help and heal patients… these have always been my typical motivations. But, since Gabe came along, I’ve learned there’s even more to it. Now I have
someone to teach and hold me accountable for being my best for the rest of my life, I look forward to it.

Not every Harvard Professor would roll the dice on a local hockey player from a small school, my as adviser did. I don’t think I was a slouch as an undergrad, but with so many well-accomplished students applying to Harvard, I didn’t view myself as anything exceptional; I guess he saw something in me that I didn’t. The first time I spoke to Kit was on the phone during my senior year. Kit called and asked if he was interrupting hockey practice and then proceeded with an inquisition of my mathematics skills at the time… I had no idea what to make of it. My first semester, Kit barred me from the lab and gave me a list of the hardest courses he could think of to take, I later learned, ‘to see if I could make it.’ Let’s just say it wasn’t the easiest transition into graduate school, but what I didn’t know at the time was that it was the transition that I needed. Over the years, I’ve learned more from Kit than any professor or mentor that I have ever had, albeit in unconventional ways at times. Kit has taught me a standard a science that will continue to impact all of my research and work going forward. He has shown me how to dissect problems and develop creative solutions. We’ve applied these skills and a drive to be innovative to heart valves as I have written about here, but there has been so much more. I’ve studied social network analysis, counter terrorism, and even fashion with Kit. Doing uncomfortable things has become the new norm. Of course, none of this would have been possible without the group. Kit will be the first to say it; it is not always (ever?) easy working in his lab. The teams of current and former graduate students and post docs that have gone through this with me have been invaluable motivationally, scientifically, and most of all, in the comradery that we have had — thank you all.
1 Introduction

1.3 million children worldwide are born with congenital heart disease every year [1], with valve malformation among the most common abnormalities. Many of these patients will require numerous surgical revisions and replacements of their heart valves as they grow. Additionally, millions of patients suffer from acquired heart valve diseases. Over 15 million people worldwide suffer from rheumatic heart disease which can cause irreversible stenosis and regurgitation of heart valves [2, 3]. Furthermore, drug-induced cardiac valve fibrosis caused by some weight loss, Parkinson’s Disease, and migraine medications (as well as some recreational drugs) have also been shown to cause severe valve thickening and malfunction [4]. These acquired heart valve diseases cannot be detected in patients until maladaptive remodeling of leaflet extracellular matrix (ECM) has occurred to a degree in which it begins to negatively affect cardiac functionality. In particular, maladaptive remodeling of the structural fibrosa layer of the valve leaflets in either congenital or acquired valvulopathies generally results in cumulative fibrotic and eventual calcific matrix deposition [5, 6]. This results in diminished and irreversible function, often requiring complete replacement of the leaflet tissue.

The standard of care for severe congenital or acquired heart valve disease is replacement with either mechanical or bioprosthetic valves. Mechanical valves are generally durable but require daily anticoagulation therapy, thus limiting the patient’s quality of life. Although bioprosthetic valves can be used without anticoagulation therapy, thus enabling a more active lifestyle for the patient, these valves need to be replaced after ~15 years due to deterioration of the thin, fixed tissue of which they are composed. Furthermore, animal sourced bioprosthetics typically require immunosuppressive therapy
[7, 8] which can result in systemic complications that are particularly harmful to pediatric patients such as nephrotoxicity, neurotoxicity, gastrointestinal toxicity, and chronic infection [9, 10]. Because neither mechanical nor bioprosthetic valves grow or adapt, children requiring valve replacement will likely undergo numerous surgeries associated with significant recovery and daily anticoagulant or immunosuppressive therapy throughout their lifetime. To overcome the limitations of conventional valve replacement, tissue engineered heart valves are being developed as permanent, regenerative replacement solutions [11-13]. However, the logistical and financial complexities associated with traditional tissue engineering methods have limited the translation of these replacement solutions which still remain largely pre-clinical.

As a result, we have identified the current methods of screening for and treatment of heart valve fibrosis to be insufficient for preventing disease and permanently treating patients who are afflicted by it. We propose that by modeling the healthy valve leaflet ECM, valvular tissues can be engineered and assembled both in vitro and in vivo as disease screening and regenerative replacement strategies respectively. In particular, we suggest that ECM-inspired organ on chip (OOC) technologies can be used to detect drug-induced valvulopathies while fibrous, degradable scaffolds can be tailored to for translatable, functional valve replacement. Herein we review the engineering design, build, and test criteria for both OOC (Section 1.1) and tissue engineering fiber production (Section 1.2) that guide our development of such technologies for semilunar heart valve fibrosis screening and repair applications.
1.1 Engineering Models for Drug Testing with Organs on Chips

1.1.1 Pharmaceutical Demand: Modeling the Patient In vitro

Bringing a drug to market is estimated to cost over $1.3 billion, a significant contributor to pharmaceutical research and development costs in the United States that exceed $50 billion annually [14]. However, despite this rising investment in drug discovery, the rate of FDA drug approval has remained constant over the last 60 years [15]. This stagnation and the rising costs of drug discovery are in part due to the failure of drug development tools and techniques to evolve with our advances in basic science.

The pharmaceutical industry often identifies toxicities in drug candidates during clinical trials [16]. The late discovery of potentially harmful effects or lack of efficacy is not, however, due to a lack of funding for early drug screening. It is estimated that nearly 32% of the $1.3 billion required to bring a drug to market is spent on pre-clinical screening and testing [17]. The high rate of late-stage drug failure may be due to ineffective screening methods that do not adequately replicate the patient. Traditional cell culture methods do not accurately model organ microenvironments and the systemic effects of drugs. Similarly, animal models do not always fully recapitulate the effects of pharmacological agents in humans. It has been estimated that about a third of successful animal studies have translated to successful human clinical trials; this is in large part due to different methodologies used in animal testing vs clinical trials, biased animal study reporting, and the physiological differences among species [18, 19].

There are a number of examples of preclinical successes that have been not only ineffective, but deadly in the clinic. Early animal models for Class I antiarrhythmic agents, including Encainide and Flecainide (Tambocor), suggested that these drugs were effective
Suppression of irregular cardiac pacing [20]. However, the Cardiac Arrhythmia Suppression Trial (CAST) of the late 1980’s later showed that patients taking Encainide and Flecainide had a 2.5 times greater risk of suffering a fatal cardiac event [21]. Similarly, early successes in canine and rodent animal models were followed by the clinical failure of the Hepatitis B drug Fialuridine (FIAU), causing the death of a third of the patients involved in a 1993 clinical trial [22]. More recently, despite evidence that the cancer drug Targretin reversed plaque build-up in mouse Alzheimer models, it proved ineffective in human Alzheimer patients [23]. These costly, unsuccessful clinical trials have motivated the pharmaceutical industry to reevaluate the methodologies used to develop drugs; successes and failures alike need to be identified early in the preclinical stages of drug discovery.

Preclinical evaluation methods might be more effectively designed if they model the patient. Patient-derived induced pluripotent stem cells (iPSCs) provide the patient-relevant foundation for in vitro disease modeling and drug testing; iPSCs are customizable cells that have the distinctive characteristics of the patient such as genetics, sex, age, and ethnicity [24]. However, the platforms in which iPSCs are used must likewise mimic the dynamic, three dimensional structure of the tissues being modeled to achieve meaningful function. To accomplish this, Organs on Chips (OOCs) are the tools being designed to recapitulate the patient by mimicking the structure, function, and subsequent response to drugs or other foreign stimuli [25]. For example, a recent study reported a model of pulmonary edema using a “lung on a chip” which was able to screen for the functional effects of a known pathogen and mimic animal testing results of a new, potentially therapeutic agent to treat the disease [26]. Clinical observations and data-
driven design, and subsequent build, of OOCs whose readouts ultimately need to register with traditional clinical diagnostics (Figure 1.1). Organs on chips can provide the pharmaceutical industry with patient-relevant drug testing models if their design, build, and test reflect the successful replication of the treated patient.

1.1.2 Design: Creating the Spatiotemporal Blueprints of Organs on Chips

The goal of OOCs is to better model the patient by recapitulating the necessary
structure-function relationships required to mimic the healthy and disease state of the afflicted organ. This is accomplished by using a multi-scale approach to recapitulate organ microenvironments in health and disease [27, 28]. Recapitulating the microenvironments of organs requires design of the extracellular matrix, definition of the biochemical environment, engineering of biotic/abiotic interfaces \textit{in vitro}, and control over the dynamic mechanical stimuli experienced in the organ [29]. However, the fundamental building blocks of tissues and organs are specialized, collectively functioning populations of cells. Mono-culture models represent an isolated and limited functionality; without the signaling and metabolic interactions with other cell types. For example, much of the research on Diabetes Types I and II is exclusively focused on the insulin producing pancreatic beta-cells [30, 31]. However, since the other pancreatic islet cell types (alpha-cells, delta-cells, etc.) and the pancreatic acinar cells are closely linked to beta-cells through their differentiation and structural proximity, it is inevitable that their interactions are functionally coupled [32]. Similarly, all organs are vascularized; the endothelial cells that comprise the vasculature are known to communicate with and influence the development and function of tissues [33]. Eventual development of OOCs with heterogeneous cell demographics will be necessary to recapitulate the dynamic interactions that potentiate organ function.

\textit{Function}, however, is derived from \textit{structure}. For many tissues, the details of the microenvironment are poorly distinguished and therefore the mapping, or physiological blueprints, of organs is difficult. To create a better blueprint, traditional methods such as autopsy and histology can provide a first approximation of structure. However, these techniques are static representations of processed samples, lacking the ability to capture
the dynamic nature of living tissue. The adaptive behavior of living tissues may be measured using non-destructive, real-time imaging techniques such as those normally used for spatiotemporal clinical diagnostics. Cardiac MRI, for example, is now capable of real-time imaging of heart-beats up to 50fps and Magnetic Resonance Spectroscopy (MRS) has been used in concert with MRI to monitor choline and lipid levels in brainstem glioma [34]. Imaging techniques such as these offer greater insight into the spatiotemporal structure and composition of tissues, providing a more detailed, multidimensional map for the design of OOCs.

The detailed mapping of individual tissues or organs is, however, insufficient to model the patient. OOC design is enabled by considering the pharmacokinetics and pharmacodynamics (PK/PD) of a drug in a multi-organ system. The PK/PD of a drug describes both how a drug or toxin is transported, metabolized, impacts targeted and non-targeted tissues, and is eventually excreted. Drug metabolism by the liver, clearance via the kidneys, or absorption through the intestines, for example, play critical roles in the PK/PD of many drugs [35]. Designing systems of interconnected OOCs to better model multi-organ systems in an effort to replicate patient PK/PD promises to be a challenge requiring a unique partnership between OOC designers and modelers.

Should connected OOCs be scaled by allometry, functional output, or simply by surface area or volume? Accurate scaling of connected OOCs is essential for the proper PK/PD of drugs in these systems. However, how to best scale OOCs still remains in question [36, 37]. Mathematical models done in silico are the design tools that can help to address the scaling and design of in vitro OOC systems [38]. By creating “biosimulations” that model drug PK/PD based on our knowledge of cellular molecular pathways, insight
into targeting complexes, metabolism, transport, and the spatiotemporal functional effects of drugs can be gained in a “virtual patient” population [39]. Only by mathematically understanding the systems and interactions being modeled in OOCs can we appropriately scale the lessons learned from trials with OOCs.

1.1.3 Build: Choosing the Materials and Tools for Building Organs on Chips

Building materials and manufacturing techniques used to put the blueprints of OOCs into practice must also be founded in patient physiology. OOCs need to be built with materials and techniques that can mimic the microenvironment of the patient. While many current OOCs replicate some of the characteristics of the tissue they aim to model, the materials and tools used to build the next generation of chips must imitate the mechanical, chemical, and structural properties of a tissue they model.

The building materials of OOCs are the cells and biomaterial-based structures that collectively mimic the organ microenvironment. The development of iPSCs as a renewable source of customizable, patient specific cells has provided a promising cell source with other human cell lines that are commercially available to build OOCs around [40, 41]. In contrast, although polydimethylsiloxane (PDMS) has served as the principal extracellular matrix (ECM) building material in current OOCs because of its low cost, optical properties, and widespread use in soft lithography for protein patterning [42, 43], it often inadequately models the tissues it is designed to mimic. PDMS, while a very useful material that has enabled the field of soft lithography since its genesis, suffers from a wide range of issues including its high stiffness and the transient nature of surface modifications which limit the length of time cells remain viable cultured on it [44]. Most problematic, however, is the absorption of proteins, drugs, and hydrophobic molecules into PDMS which complicates
the PK/PD and accurate dosing of drugs [45]. The use of PDMS puts a significant limitation on the models we can achieve with OOCs.

Alternatives to PDMS may offer an opportunity to mimic organ microenvironments on OOCs while evading the difficulties described above. Hydrogels that are mechanically tunable and chemically functionalized for cell attachment, such as alginate or gelatin, are an alternative building material to PDMS that better mimic soft tissue microenvironments and enable long term culture [46]. Native ECM protein-based hydrogels assembled in extruded micro-tubes or fibers have been shown to guide aligned tissue formation of a variety of cell types in vitro [47]. Similarly, protein or blended protein-synthetic polymer nanofibers are also chemically and mechanically tunable substrates with nanoscale features and alignment similar to native matrix [48, 49]. With these alternative materials, we can begin to model more complex physiological states on OOCs. Models of fibrosis in the heart, for example, can be achieved in vitro by mechanically altering the ECM stiffness [50] or dynamic stretch [51] of tissues. Similarly, by chemically functionalizing ECM with specific proteins and/or tuning the stiffness of the substrate, stem cell differentiation can be guided to study development [52, 53]. Changes in extracellular matrix composition and mechanics influence and result from pathophysiological behavior of tissues; by controlling these properties of the ECM materials, we may better control the pathophysiology in OOCs.

OOC assembly requires a toolset of fabrication techniques suitable for mass manufacturing. The development of soft lithography has led to microcontact printing of substrates to control cell and tissue structure in vitro [54, 55] as well as inexpensive and relatively simplistic manufacturing of microfluidics for drug discovery and cell culture [56, 57]. These techniques, though suitable for the laboratory, are largely manual and require
scalable automation to meet the requirements of mass manufacturing. However, recent advances in 3D printing techniques have enabled the direct fabrication of microvasculature and tissues using a number of synthetic and biological polymers printed with and without, embedded cells [58, 59]. Manufacturing tools, such as 3D printing, that can simultaneously create the structure of an OOC while also organizing its cellular components, can bring OOCs from “boutique” laboratory tools to the pharmaceutical industry.

However, manufacturing at such a large scale requires the implementation of quality controls and standards not currently established in the field. For example, if reprogrammed iPSCs are to be the model cell source for an organ, whose organ are they modeling? The genotype, phenotype, and developmental maturity of iPSC derived cell lines must be consistent and defined for us to know what the clinical comparison of the OOC is [60]. Similarly, the cell culture media for organs on chips needs to be standardized. The field still relies heavily on serum-based, specific growth factor-supplemented, and/or proprietary specialized media which makes the culture of heterogeneous cell populations or the connection of different OOCs into systems difficult. As individual OOCs are assembled into larger systems, their platforms and connections should be standardized and modularized to simplify assembly and allow for dynamic adjustments to the system. The manufacture of chips suitable for the pharmaceutical industry will require controls and standards to be established for all components of OOCs.

1.1.4 Test: Correlating Organs on Chips to the Patient

The goal of OOCs is to better mimic the human response of the patient in vitro; the readouts of OOCs must therefore map to the standard clinical diagnostics they are built to model. However, the correlation of OOC readouts to their clinical counterparts is difficult
to make due to differences in measurement techniques (Figure 1.2). Because of these differences, there remains a data fusion problem between OOCs and the clinic. How can the data we obtain from OOCs be correlated to organ physiology and pathophysiology? By mapping the more simplified readouts of OOCs to their corresponding clinical diagnostics, we may better determine their significance. Subsequently, implementing control loop engineering techniques into OOCs will enable the manipulation of OOC readouts which can, when collected in real-time, more accurately model the living and dynamic patient.

Because OOCs are simplified models of organs, their readouts are also simplified subsets of overall organ function measured in the clinic. The result is that OOCs only tell part of the story; interpreting their readouts in a clinical context is often difficult. For example, although electro/magneto encephalography (EEG/MEG) are principally measures of extracellular electric fields and currents, the exact mapping between organ-level recorded EEG/MEG and cellular-level electrode measurement commonly done in vitro is not clear [61]. To interpret the more basic measurements of OOCs will require computational modeling to put the measurements in greater context. Data from organs on chips can inspire computational models in combination with clinical or other data and assumptions to build models of the patient. Calcium transients in a single cardiomyocyte, for example, can be used in a series of linked mathematical models to determine the resulting whole-heart function including clinical readouts such as imaging, stroke volume, ejection fraction, and cardiac output [62]. By pairing the data obtained from OOCs with appropriately scaled, mathematical models of organ function, we can better understand their relevance to the clinic.
Although the computational models used to interpret OOCs may be complex, they will be represented by equations with inputs (OOC functional data) and outputs (organ level functional data). For the outputs of these models to mimic the dynamic states of health and disease, the inputs from OOCs must be controlled by opening and accessing the feedback loops that govern the physiology [63]. Controlling OOCs in this way may offer systems capable of simulating a number of intra/extra-organ interactions [64]. However, the field is currently ill-equipped to implement such control loops on chips due to the lack of real-time access to the inputs and outputs of OOC systems. Many common methods of analysis, such staining or antibody-based assays, can be destructive and suffer from significant time delays before readout. Therefore, OOC testing must move toward enabling real-time, non-destructive, and multi-modal biochemical read-write capabilities in order to actively control and monitor the biology on the chip.

There are many technologies that may help achieve real-time control over OOC inputs and outputs. Integrated electrode arrays [65], optogenetics [66], live fluorescence imaging, and various combinations of all these techniques can be used to stimulate and extract data from OOCs in real-time. More recently developed technologies such as functionalized nanoparticles and conductive polymers incorporated into bio-systems have provided targeted delivery of drugs, antigens, and other biomolecules to both perturb and screen/image systems [67, 68]. Furthermore, connecting Mass Spectrometry (Mass Spec) and/or Ion Mobility Spectrometry (IMS) systems to OOCs can yield nearly instant insight into the molecular composition of circulating media or chip waste [69]. By controlling the
inputs of an OOC and being able to observe their effects in real-time, OOCs can be tested and treated as dynamically as the patient in the clinic.

Figure 1.2: Clinical Function Diagnostics versus Typical Organ Chip Readouts. A comparison of various clinical function tests used for diagnostics against the typical readouts that are possible with Organ-on-Chip technologies for the Brain, Heart, Liver and Intestines. Although some OOC readouts can be related to functional tests (as with the Liver), the majority lack a firm relationship with a corresponding clinical diagnostic technique.

1.1.5 Conclusion for Engineering Organs on Chips

The clinical failures of drugs have motivated a reassessment of how they are tested during the early stages of development. Organs on Chips are a developing toolset which may offer a more relevant means of drug testing to supplement standard cell culture and animal testing. The goal of OOCs is to better mimic the patient in vitro with the hope of
providing the platform for *in vitro* clinical trials and customized, patient specific drug testing. Therefore, OOCs must reflect the complexity and uniqueness of the human physiology and pathology they are designed to model. Building standards and controls for the cells, ECM and synthetic materials, and fabrication tools need to be established to ensure that OOC manufacturing is sustainable and cost efficient. Finally, the readouts obtained from OOCs must translate to the clinic. This requires computational modeling to extrapolate data from OOCs to their clinical significance. By controlling the inputs and monitoring the outputs of OOCs in real-time, this clinical significance will be a better representation of the dynamic nature of the patient.

### 1.2 Fibrous Scaffolds for Engineering Regenerative Cardiac Scaffolds

Extracellular matrix (ECM) structure and biochemistry provide cell-instructive cues that promote and regulate tissue growth, function, and repair. From a structural perspective, the ECM is a scaffold that guides the self-assembly of cells into distinct functional tissues. The ECM promotes the interaction between individual cells and between different cell types, and increases the strength and resilience of the tissue in mechanically dynamic environments. From a biochemical perspective, factors regulating cell-ECM adhesion have been described and diverse aspects of cell-ECM interactions in health and disease continue to be clarified. Natural ECMs therefore provide excellent design rules for tissue engineering scaffolds. The design of regenerative three dimensional (3D) engineered scaffolds is informed by the target ECM structure, chemistry, and mechanics, to encourage cell infiltration and tissue genesis. This can be achieved using nanofibrous scaffolds composed of polymers that simultaneously recapitulate 3D ECM architecture, high-fidelity nanoscale topography, and bio-activity. Their high porosity, structural anisotropy, and bio-
activity present unique advantages for engineering 3D anisotropic tissues. Our knowledge of the anatomy and physiology of the heart, as well as our ability to create synthetic ECM scaffolds have advanced to the point that valve replacement with nanofibrous scaffolds may be achieved in the near future.

1.2.1 Fiber-Based Scaffolds for Regenerative Tissue Engineering

In their 2002 Viewpoint article, Hench and Polak [70] described a transition to “Third-Generation Biomedical Materials” that stimulate specific cellular responses to promote endogenous tissue regeneration and help the body to heal itself. To accomplish this goal, implanted scaffolds should first minimize toxic response in the host and subsequently recapitulate properties of the native tissue’s extracellular matrix (ECM) to promote cell assembly into functional tissues. Mechanotransduction through the cell-ECM interface plays a fundamental role in regulating tissue homeostasis, growth, and regeneration [71-76] (Figure 1.3). In muscular organs, for example, ECM morphology and composition to form tissues.

Figure 1.3: Cellular/Tissue Mechanotransduction through the Fibrous ECM. Nanofibers incorporating bioactive components can utilize the natural adhesion network of cells to serve as a biomimetic conduit for signaling between the biotic interface of the cell and the abiotic interface of the scaffold. Cellular integrins bind to both the intracellular cytoskeletal and microtubule networks and to the bioactive nanofiber scaffold mimicking the native ECM structure and composition to form tissues.
elasticity regulate cell shape and coordinate myofibril assembly, thereby influencing tissue architecture and contractile strength [77-80]. Specifically in the heart, a fibrillar ECM network provides guidance cues that direct the spatial and temporal synchrony of cardiac development. Thus, recapitulation of this ECM network using fibrous materials may be a crucial design consideration of engineered cardiovascular tissues.

The use of fibrous cell culture substrates to study tissue regeneration can be traced back at least a century to the work of Ross Granville Harrison who, in 1914 [81], cultured embryonic frog and chick cells on spider silk, noting that “the solid support influence[d] the form and arrangement assumed by the moving cells” and cells were “arranged with reference to the web fibers, and they [were] usually drawn out into long processes”. Contact guided cell growth was subsequently studied on diverse substrates (e.g., glass fibers [82], oriented collagen [83] and micropatterned features [84]) but predictable tissue assembly required discovery and classification of tissue-specific structures, cell types, cell adhesion proteins [85-88], and their interactions with the extra-cellular microenvironment [73, 89, 90]. Extensive study of these components and properties of cell-ECM interaction provide a mechanistic understanding of tissue self-assembly that can be incorporated into the design specifications of engineered tissues to guide the development of more physiologically-relevant cellular scaffolds [91-93]. Scaffolds composed of fibrous materials are increasingly used for regenerative medicine because manufacturing platforms now exist capable of producing fibers with a wide range of structural and biochemical properties [94-98]. Fiber scaffolds fabricated using these techniques can mimic the native ECM and be woven or otherwise assembled into organ-scale structures with adequate porosity and structural stability to support cell infiltration and assembly [99]. Moreover,
the incorporation of bioactive molecules into synthetic fibrous scaffolds, such as native ECM components and growth factors, may enhance the development of engineered tissues into more accurate tissue analogs and promote healthy integration into diseased or injured tissues [100].

1.2.2 Design Criteria for Engineered Cardiac Valve Tissues

The heart is a muscular pump tasked with continuously providing efficient blood transport throughout the body. This is achieved through hierarchical control of structure and function integrated over multiple spatial scales and structures: muscle, blood vessels, and valves [75, 101]. In particular the stratified, fibrous structure of the cardiac valve tissues enable the heart to function efficiently as a one-way pump; disruption of this structure can result in reduced cardiac performance. A key challenge in the field of tissue engineering is defining the standards by which successful replication of native tissue function is achieved, particularly in light of increasing demand for patient-relevant tissue models created using human stem cells. What metrics should be used to determine the success of an engineered tissue fabricated using a fibrous scaffold? Physical material properties, two dimensional planar alignment, and three dimensional global architecture are important aspects of native tissues that must be recreated in engineered scaffolds to guide cellular self-assembly. Additionally, biochemical properties, degradation kinetics, and bioactive components must be optimized to recapitulate or trigger specific in vivo responses and tissue development in engineered scaffolds meant for implantation.

In order to fabricate biomimetic tissues that recapitulate the function of the heart valves, it is first necessary to quantitatively define the relevant structural and performance attributes that define normal physiological function. Although the standard comparison for
the developed functionality of an engineered tissue is the native tissue it is designed to repair or replace, should native tissue chemical and mechanical properties also serve as design criteria for fiber scaffolds? Alternatively, should some immature or basic model of the tissue structure and composition be the standard for a fibrous scaffold: a structure and composition that will both function immediately and best initiate scaffold remodeling/tissue formation once implanted? Comprehensive, quantitative comparison of engineered tissues versus healthy, mature tissues using machine learning approaches [102, 103] and statistical metrics, such as strictly standardized mean difference, could provide robust quality assurance rubrics for determining the fitness of engineered tissues for regenerative therapy applications [60]. Traditional tissue engineering approaches involve scaffold to tissue fabrication: scaffold production, *in vitro* cell seeding, *in vitro* cell-scaffold conditioning to form tissue, and finally implantation. At each phase, metrics are defined to determine success. For example, mechanical/chemical properties of the raw scaffold, efficiency of seeding, degree of remodeling by the cells during conditioning *in vitro*, and the eventual functionality of the implanted construct.

1.2.2.1 *Cardiac Valve Structure and Function*

Cardiac valves maintain unidirectional blood flow through the heart by coordinated action of thin membranous structures known as leaflets or cusps. Key structural and functional properties of the cardiac valves are summarized in Table 1.1. Valve tissue undergoes significant remodeling throughout life and in response to environmental stimuli [104]. However, aberrant valve remodeling can lead to valvular diseases that alter blood flow and mechanical loads placed on the myocardium, ultimately disrupting broad aspects of cardiac function [105, 106]. During the cardiac cycle, increased or decreased left
ventricular pressure in systole or diastole, respectively, force the aortic valve leaflets to open and close (Figure 1.4a), a process that is repeated ~3 billion times over the course of an average lifetime [107]. Aortic valve leaflets contain three layers with collagen fibers predominant in the fibrosa, a GAG-rich matrix in the spongiosa, and elastin sheets in the ventricularis [108]. Collagen fibers within the valve leaflets are load bearing during diastole (closure) and oriented principally in the circumferential direction. Elastin fibers are responsible for leaflet recoil during systole (opening) and are principally oriented in the radial direction of the leaflet (Figure 1.4b). Disoriented ECM fibers are a hallmark of valve disease (Figure 1.4a) although their exact orientation and bundle size is distinctive to each leaflet (Figure 1.4c). To study the effects of collagen fiber alignment on valve kinematics and hemodynamics, Marom et al. [109] used numerical fluid-structure interaction models of asymmetric mapped collagen fiber networks from measurements of porcine valve and a simplified-symmetric network (Figure 1.4d). They showed that fibers carried most of the mechanical load and asymmetric internal structure had a considerable impact on the hemodynamics. Regions with less dense fiber network were subjected to higher internal stress and flow shear stress magnitudes and, therefore, are at higher risk of damage. Numerous attempts have been made to fabricate tissue engineered scaffolds that recapitulate the structure and function of native heart valves [169, 217-219]. By constructing the trileaflet global structure of semilunar valves using fibrous material, *in vitro* cell seeding can allow for physiologically-relevant conditioning that provides structural and mechanical cues for tissue formation before implantation.
Table 1.1: Structural and Functional Properties of Cardiac Valves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechanical Properties</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Native Biaxial Aortic Valve Stiffness</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIRCUMFRENTIAL (kPa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low-Strain (0-18%): 89.1±4.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid-Strain (18-28%): 825.11±29.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High Strain (28-35%): 1577.17±53.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RADIAL (kPa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low-Strain (0-18%): 33.94±1.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid-Strain (18-28%): 116.43±4.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High Strain (28-35%): 324.93±9.84</td>
</tr>
<tr>
<td></td>
<td>Native Biaxial Pulmonary Valve Stiffness</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIRCUMFRENTIAL (kPa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low-Strain (0-18%): 11.31±0.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid-Strain (18-28%): 408.23±18.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High Strain (28-35%): 1457.19±58.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RADIAL (kPa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low-Strain (0-18%): 11.67±0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mid-Strain (18-28%): 50.16±2.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High Strain (28-35%): 172.44±5.24</td>
</tr>
<tr>
<td>Aortic Valve Flow Rate (systole, healthy)</td>
<td>1.35±.035 m/s</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>(highest flow rate of valves)</td>
<td></td>
</tr>
<tr>
<td>Mitral Valve Flow Rate (diastole, healthy)</td>
<td>0.89±0.15 m/s</td>
<td>[112]</td>
</tr>
<tr>
<td>Pulmonary Valve Flow Rate (systole, healthy)</td>
<td>0.6-0.9 m/s</td>
<td>[113]</td>
</tr>
<tr>
<td>Tricuspid Valve Flow Rate (diastole, healthy)</td>
<td>0.7 m/s</td>
<td>[114]</td>
</tr>
<tr>
<td></td>
<td>(lowest flow rate of valves)</td>
<td></td>
</tr>
<tr>
<td>Max Closing Strain Rate (diastole, loaded)</td>
<td>Peak Strain Rate: 1000%/s</td>
<td>[115]</td>
</tr>
<tr>
<td>Peak Tension Values (general valve tissue range)</td>
<td>Peak Loading Range: 50-100 N/m</td>
<td>[116]</td>
</tr>
<tr>
<td>Transvalvular Pressures (back pressures)</td>
<td>Aortic Valve: 80mmHg</td>
<td>[116]</td>
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<tr>
<td></td>
<td>Pulmonary Valve: 10mmHg</td>
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</tr>
<tr>
<td></td>
<td>Mitral Valve: 120mmHg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tricuspid Valve: 25mmHg</td>
<td></td>
</tr>
<tr>
<td>Valve Area Normal</td>
<td>Aortic Valve: 4.6±1.1cm²</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>Mitral Valve: 7.8±1.9cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pulmonary Valve: 4.7±1.2cm²</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tricuspid Valve: 10.6±2.6cm²</td>
<td></td>
</tr>
<tr>
<td>Shear on Aortic Valve (aortic surface, systole)</td>
<td>20 dyn/cm²</td>
<td>[118]</td>
</tr>
</tbody>
</table>
### Table 1.1 (Continued)

<table>
<thead>
<tr>
<th>ECM Properties</th>
<th>Morphology: Characteristic cobblestone morphology <em>in vitro</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endothelial Cells</strong></td>
<td></td>
</tr>
<tr>
<td>(stain and western)</td>
<td>Immunohistochemistry and Western:</td>
</tr>
<tr>
<td></td>
<td>1) vWF- granular and perinuclear</td>
</tr>
<tr>
<td></td>
<td>2) Vimentin- uniform distribution</td>
</tr>
<tr>
<td></td>
<td>3) DAPI- large and rounded nuclei</td>
</tr>
<tr>
<td>No Staining:</td>
<td></td>
</tr>
<tr>
<td>1) αSMA</td>
<td></td>
</tr>
<tr>
<td>2) Myosin and desmin</td>
<td></td>
</tr>
</tbody>
</table>

| **Interstitial Cells**  |
| (stain and western) | Morphology: Mixture of spindle and rhomboid shaped cells, rapid proliferation *in vitro*  |
| | Immunohistochemistry and Western:  |
| | 1) Vimentin- strong and uniform distribution (fresh & culture)  |
| | 2) αSMA- strong staining (in culture, not fresh)  |
| | 3) DAPI- large and rounded nuclei  |
| | 4) Weaker stains for myosin, calponin, and h-caldesmon (not seen in fresh explants)  |
| No Staining:  |
| 1) Desmin  |
| 2) vWF  |

| **Native Matrix Composition**  |
| (histology) | 1) Vimentin uniformly expressed throughout valve layers  |
| | 2) αSMA, myosin, and calponin only found in ventricularis  |
| | 3) Single cell layer endothelialization (vWF)  |
| | 4) Collagen primarily in the fibrosa, arranged parallel to the free edge of leaflet (circumferentially aligned); 10-80µm depth; loading during diastole  |
| | 5) Elastin primarily in the ventricularis, arranged perpendicular to the free edge of the leaflet (radially aligned); 5-30µm depth; elastic recoil during systole  |
| | 6) Hydrated glycosaminoglycans in spongiosa, ‘lubrication’ for the fibrosa and ventricularis layers  |

[119]  
[116]
Figure 1.4: Structure of the Aortic Valve in Health and Disease. (a) Schematic description of a healthy aortic valve. Systolic contraction of the left ventricle forces the aortic valve leaflets to open, allowing blood to enter the aorta. The reversed pressure gradient, created when the heart rests in diastole, causes the aortic valve leaflets to close, preventing retrograde blood flow into the heart. Circumferential collagen alignment allows the leaflets to stretch in the radial direction while providing the tensile strength required to prevent leaflet prolapse. Loss of ECM organization associated with fibrocalcific diseased state. Reprinted from [120] with permission from Macmillan Publishers Limited; (b) Schematic cross-sectional view of a valve leaflet showing fibrosa, F, spongiosa, S, and ventricularis, V, layers. Circumferential collagen alignment in the fibrosa and radial elastin alignment in the ventricularis are indicated; (c) Left: Stretched cusps and the mapped collagen fiber network. Right: A 3D view from the aortic side of collagen fiber maps and associated maximum principal stress contours plotted on the deformed structure during diastole. Reprinted from [109] with permission from ASME.
Cardiac valves exhibit pronounced structure-function relationships that are often disrupted in valvular heart disease (VHD) and result in abnormal valve leaflet number, morphology, mechanical properties, and/or biochemical composition [121-123]. For example, calcific aortic valve disease initiation is associated with fibrotic collagen accumulation and disorientation, which leads to thickened, stiffened valve leaflets and a deterioration in function [120]. Valve leaflets contain valvular interstitial cells (VICs) and the leaflets are coated by a monolayer of valvular endothelial cells (VECs) that are in contact with circulating blood. VICs are associated with remodeling and repair [124] but their dysregulation can lead to leaflet fibrosis and calcification that progress to sclerosis and stenosis [105, 125]. VECs suppress VIC pathological differentiation into chronically α-SMA positive myofibroblasts, an effect that is enhanced by exposure of the endothelium to flow-induced shear stress [126]. VECs also undergo endothelial-mesenchymal transformations during valve development and may replenish VIC populations during adulthood [127]. For these reasons, there is hope that endothelial cell recruitment to valve scaffolds can lead to functional VEC leaflet coating and EMT-based repopulation of interstitium. Because circulating endothelial progenitors are rare [128], a strategy based on endothelial recruitment from vascular structures adjacent to the implanted scaffold appears favorable. Fibrous scaffolds may provide structural cues required to initiate endothelial cell recruitment either from circulating blood or from the border zone adjacent to implanted scaffolds. Fibrous scaffolds may provide structural cues required to initiate endothelial cell recruitment and serve as a substrate to recapitulate the valve interstitium for VIC regulation.

1.2.3 Fibrous Scaffold Production Techniques
Nanofibers with high surface area to volume ratios and small diameters mimic ECM structure, thus providing suitable substrates for regenerative tissue growth. Three prominent nanofiber scaffold production methods are illustrated in Figure 1.5 including decellularization of tissues (Figure 1.5a), electrospinning (Figure 1.5b), and Force Spinning (Figure 1.5c). The respective advantages and disadvantages of these methods are outlined in Table 1.2. Structural properties such as fiber diameter size, thickness, and porosity are tuned in these scaffolds to allow cellular integration and tissue formation. The biomechanical composition of scaffolds is tailored to recapitulate the native tissue chemistry and mechanics as instructive cues for specific tissue formation. For example,

**Figure 1.5: Strategies for Building Fibrous Scaffolds.** (a) Decellularized human heart (top), reprinted from [129] with permission from Elsevier, and decellularized porcine submucosa (bottom), scale bar is 500nm. (b) Electrospun fibrous use high voltages to extrude conductive fiber precursors, scale bar is 30μm, reprinted from [130] with permission from Istituto Superiore di Sanità. (c) Force spinning methods such as rotary jet spinning adopt many of the benefits of electrospinning while scaling up production levels and expanding material diversity, scale bar is 30 μm.
heart valve tissue scaffold porosity must be tuned to allow cell infiltration but limit vascularization. An elastic material (i.e. elastin) oriented radially will provide scaffold recoil and a strong material (i.e. collagen) oriented circumferentially will provide structural integrity in valvular scaffolds. Although decellularized tissues and organs provide 3D substrates for tissue regeneration, the lack of experimental control over constituent components hinders reproducibility of scaffold composition and reduces predictability of cell response to the scaffold. Emerging fiber production techniques aim to overcome these limitations using a “bottom-up” approach to scaffold engineering where each scaffold component (e.g., ECM bioproteins) and structure is precisely controlled.

Table 1.2: Fibrous Scaffold Fabrication Techniques

<table>
<thead>
<tr>
<th>Scaffold Fabrication Technique</th>
<th>Design Advantages</th>
<th>Manufacturing Limitations</th>
</tr>
</thead>
</table>
| Decellularized Tissues and Organs | • Purely extracellular matrix  
• Exact multiscale structure | • Immature cells within a mature matrix  
• Non-uniform decellularization protocols  
• Lack of standards for successful decellularization  
• Variable sample composition  
• Variable sample quality and sourcing |
| Electrospinning | • Diversity of materials and solvents  
• Control of fiber morphology (nano to macro)  
• Nano-micro scale fiber | • Requires conductive polymers and solvents  
• Reliance on high voltage for fiber formation  
• Low production rates  
• Reproducible fiber production requires environmental control |
| Force Spinning  
(Rotary Jet Spinning) | • Diversity of materials and solvents  
• Control of fiber morphology (nano to macro)  
• Nano-micro scale fibers  
• Moderate production rate | • Reproducible fiber production requires environmental control |
1.2.3.1 Decellularized Extracellular Matrix Fibrous Scaffolds

The decellularization of allogeneic or xenogeneic tissues may be the most structurally and compositionally relevant method to fabricate fibrous scaffolds for regenerative tissue engineering (Figure 1.5a). A fully decellularized tissue, in theory, will retain the fibrous, three dimensional structure and organization of its constituent protein ECM providing the exact scaffolding framework of a specific organ [131]. Decellularizing tissues is rooted in the successes of transplanted cadaveric and donor tissues; by further removing the native cells of a transplant tissue, the aim is to avoid the risk of rejection and be able to “customize” the tissue with a different native or stem cell based population of cells [129]. Physical, chemical and enzymatic treatments are used to decellularize tissues [132, 133], including lungs [134], kidneys [135], heart [136], bone [137], and vasculature [138]. Recently, a detergent based treatment protocol using both antegrade and retrograde perfusion through the native tissue vasculature at low relative physiological pressures was published for the effective decellularization of whole hearts, lungs, and kidneys [139]. The maintained vasculature of decellularized tissues provides not only the necessary access to the tissue for decellularization, but also serves as a recellularization conduit and nutrient transport system in the regenerating tissue. Reseeded, whole heart scaffolds produced by perfusion methods have even been shown to develop immature organ-level function in vitro such as electrical conduction and mechanical function (pumping/contraction) of the reseeded heart [140, 141]. The return of some organ level function demonstrates that decellularized tissues may provide a viable source of fibrous scaffold with the architectural and compositional cues necessary to potentiate tissue regeneration.
Although decellularized tissues and organs can provide fibrous scaffolds that resemble native structure and biochemical composition, matching these properties to specific patient needs is challenging. The matrix necessary and sufficient to activate immature resident stem cell populations and regulate the development of these cells into mature tissues remains unclear and is not controlled experimentally when using developmentally mature decellularized tissues. Furthermore, there exist many unique processing methods that attempt to sufficiently decellularize the tissue while minimizing ECM deterioration and aberrant host inflammatory responses [142, 143]. This diversity in the field has resulted in a lack of minimum standards for determining successful decellularization and host reactions to organ- or tissue-based fibrous scaffolds [132].

1.2.3.2 Synthetically Produced Fibrous Scaffolds

Synthetic fibrous scaffold production and evaluation can be subject to higher degrees of manufacturing control than tissue decellularization. This lends itself to the introduction of good manufacturing procedures and increased reproducibility and reliability. The most popular method of fabricating nanofiber scaffolds via synthetic engineering is electrospinning (Figure 1.5b). Electrospinning is the process by which an electrically charged polymer melt or solution is extruded through an orifice, creating a jet that solidifies into nanofibers that are subsequently collected on a substrate [144]. Numerous methods now exist to electrospin nanofibers, including traditional needle arrays and needle-free techniques [94, 95] developed to improve fiber formation and production rates: these include bubble electrospinning [145] and microfluidic electrospinning [146]. Synthetic polymers and biological proteins are electrospun using these techniques independently [147, 148] or in combination [149, 150]. The diversity of electrospinning techniques and
electrospun materials has translated to numerous fibrous tissue engineering applications including ligament, tendon, skeletal muscle, skin, blood vessel, and neural scaffolding [151].

The diversity of electrospinning techniques and applications result from controlled engineering design and fabrication processes. Unlike decellularized tissue scaffolds manufactured using top-down approaches, synthetic electrospun scaffolds are built “bottom-up.” Because of this “bottom-up” approach, scaffold characteristics such as fiber diameter [152], fiber alignment [95], scaffold porosity for cell infiltration [153], and macroscopic scaffold geometry [154] can be controlled by simply varying needle diameter, applied needle voltage, flow rate or viscosity of solution/melt, along with a number of other spinning parameters [155]. In addition to control over these physical characteristics, electrospun scaffolds can be bioactively functionalized with the inclusion of drugs [156] or specific growth factors [157] within the fibers to guide host response and endogenous repair mechanisms. Recently, electrically conductive fibers [158] and metabolic sensors [159, 160] have been incorporated into ‘smart’ electrospun scaffolds for real-time tissue performance monitoring. Since electrospun fibers are built by a “bottom-up,” fiber-by-fiber approach, they can be biomechanically tuned and functionalized for specific tissue scaffolding.

However, it is also this fiber-by-fiber approach to creating scaffolds with electrospinning that limits its utility as a manufacturing method. Production rates of electrospun fibers are very low compared to industrial fiber production techniques; the most productive industrial scale electrospinning systems hardly reach kilograms of fibers per hour whereas common industrial scale melt or wet spin fiber production can reach tens
of tons of fibers per hour [161]. Although numerous multi-needle setups have been developed to increase fiber production rates [162], these methods complicate the normally simple electrospinning setup which makes it an attractive manufacturing technique by introducing multiple electric fields and numerous high voltage connections. Additionally, because of this high voltage required to electrospin, material and solvent choice for electrospinning is limited to polymers that are soluble in conductive solvents. This is particularly restrictive for electrospinning regenerative scaffolds made of or including protein due to the detrimental effects of voltage and solvent on protein three dimensional structure [163]. Melt-electrospinning processes have been developed to address the limitations of solvents [164], though the resultant fibers generated are commonly on the order of microns in diameter and the high temperature of the melt process likely has a denaturing effect on electrospun proteins and stability of synthetic polymers. Production rate and material manufacturing limitations have limited the translation of electrospun fibrous scaffolds to industrial-scale production.

In order to overcome the manufacturing limitations of electrospinning, force-based fiber fabrication via Rotary Jet Spinning (RJS) [97] was developed for the fabrication of fibrous scaffolds (Figure 1.5c). RJS uses the centrifugal forces developed in a rotating reservoir perforated with a single or multiple micron-scale orifices to circumferentially extrude nanofibers from solution. By varying polymer solution and spinning parameters (e.g. solution viscosity, rotation rate and extrusion speed), nanofiber properties such as fiber diameter and alignment can easily be controlled using the RJS fabrication technique [165]. Production rates achievable by a single force driven system (~100 g/hr) are roughly two orders of magnitude higher than similarly scaled electrospinning systems. Collagen
and gelatin nanofibers are rapidly fabricated with the RJS both with and without mixing with synthetic polymers to tune mechanical properties and degradation profiles of scaffolds [98]. In addition to collagen and gelatin, the high shear forces developed in the orifice of the RJS reservoir during extrusion has been exploited to initiate fibrillogenesis of beta sheet rich proteins such as silk to produce insoluble, pure protein nanofibers without the need of post-processing crosslinking [166]. Overcoming the production rate limitations of electrospinning while maintaining, and even increasing, the versatility of spinning materials, RJS and similar force spinning techniques [167] bring synthetic nanofiber based scaffold fabrication processes closer to true manufacturing and clinical translation.

The challenge of any synthetic fiber fabrication technology is building the global, three dimensional structure of the target cardiac tissue; that is, the three dimensional collection of fibers. Because of the “bottom-up” approach of synthetic fibrous scaffold fabrication, building from the nano and micron scales of single fibers to the macro-scale geometries of scaffold tissues requires innovative, cumulative fiber collection techniques. Using electrically grounded plates, cylindrical mandrels, and other specially shaped columnar collectors for example, various geometries can be manufactured via electrospinning fibers to achieve specific tubular fibrous structures. These innovative fiber collection strategies suggest that full multiscale recapitulation of tissue and organ structures will soon be possible using nanofiber production platforms such as electrospinning and force spinning.
1.2.4 Cardiac Valve Tissue Repair Using Fibrous Scaffolds

The overall strategy for designing synthetic cardiac tissues using bio-inspired nanofibrous scaffolds is summarized in Figure 1.6. Given the complexity involved in assessing the quality of traditional tissue engineered products that are seeded, cultured, and conditioned \textit{in vitro}, how practical—logistically—is their translation to common usage in the clinic? There are currently no standardized guidelines for determining the fitness of engineered heart tissues, although attempts to use computer algorithms relying on gene expression profiles, structural phenotyping, and statistical combination of structural and functional measurements have been proposed to allow reliable, quantitative comparison [60, 102, 103, 168]. The appropriate cell sources at the necessary seeding densities must be identified, customized bioreactors built, optimization of conditioning parameters established, and standards for desired tissue growth established before implantation can be attempted. This level of \textit{in vitro} customization results in high cost and time to clinic for a tissue engineered scaffold [220-222], limiting the translation of the process and product. Traditional tissue engineered scaffolds that are seeded with cells \textit{in vitro} are evaluated by a number of biological parameters such as how the cells adhere to and penetrate the scaffold, what and when phenotypic changes occur, the metabolic activity of the cells/tissue, and how the cells/tissue function \textit{in vitro} [169]. How these parameters change over time depends on the scaffold, cells, culture conditions, bioreactors, and time in culture which are unique to each application and are likely to change dramatically post implant. By focusing on cell-free scaffolds, we can apply well defined quality metrics to the scaffold itself [223, 224]. Scaffold qualities that include mechanics, biochemical composition, porosity, fiber alignment, three dimensional architecture, and cargo release profiles can be
precisely and quantitatively measured. Fabricating cell-free scaffolds from the ground up (i.e. using as few biochemical components as are necessary) not only provides precisely measurable quality control metrics but also simplifies the host-response problem by narrowing the parameter space.

Figure 1.6: Overview of Bio-Inspired Cardiac Fibrous Scaffold Fabrication.

Native cardiac tissue structure and function provide design criteria for engineered fibrous scaffolds. Synthetic vessel, valve, and myocardial scaffolds have each been produced: (i) Electrospun 3D nanofibrous tubes with controllable architectures. Reprinted from [170] with permission from American Chemical Society Publications. (ii) Electrospun trileaflet valve scaffold. Reprinted from [171] with permission from Elsevier; (iii) Confocal micrograph electrospun scaffolds seeded with H9C2 cardio-myoblasts demonstrating cell alignment (Green: F-actin (phalloidin), blue: nuclei (DAPI)), inset shows an atomic force microscopic (AFM) image of the scaffold's 3D surface topography. Scanning area is 50 x 50 μm. Reprinted from [172] with permission from Elsevier; (iv) Valve interstitial cells infiltrating electrospin scaffolds (SEM colored). Reprinted from [173] with permission from Elsevier.
1.2.4.1 Fibrotic ECM Structure Following Injury or Disease

Scaffolds designed for cardiac repair aim to improve upon the body’s limited endogenous repair mechanisms. Heart failure is often associated with tissue fibrosis and scarring [174] and fibrosis throughout the cardiovascular system leads to maladaptive remodeling and inefficient function of myocardial, valve, and vascular tissues. Fibrosis in the myocardium due to infarct can cause hypertrophic expansion of the ventricles leading to poor contractile efficiency and reduced cardiac output. Fibrosis of the cardiac valves can lead to calcification, resulting in stenosis and/or insufficiency and reduced cardiac output. Repairing and preventing the structural remodeling resulting from these pathological conditions requires that synthetic scaffolds provide the biological cues necessary to direct cellular activity in a manner that inhibits or reverses scar formation.

1.2.4.2 Fibrous Scaffolds for Valve Replacement

In contrast to mechanical or bioprosthetic heart valves, fibrous constructs preserve multiscale features of natural valves and possess the ability to promote endogenous remodeling. These 3D fibrous valve scaffolds support cell culture and matrix remodeling in response to physiologically-relevant flows and pressures when cultured in vitro [175] or in vivo [176]. Implantation of non-endothelialized heart valve constructs into non-human primates resulted in nearly confluent endothelialization after just 4 weeks in vivo [177], suggesting that endothelialization prior to implantation may not be necessary. This is important because reducing long-term in vitro culture and conditioning simplifies design and testing and reduces risk of infection. Engineered valve scaffolds that are either seeded with patient-derived stem cells or implanted cell-free may overcome the limitations of immunogenic rejection reported for xenogenic or allogenic transplants. Importantly, they
are not dependent on the availability of healthy human donor tissue and may be tailored to patient-specific needs. This is especially true for cell-free scaffolds for which informed design, based on patient data, scaffold fabrication and implantation can be achieved in less than a day. It may also be possible to implant these scaffolds using minimally invasive procedures. For example, Weber et al. [175] confirmed that the structure of tissue engineered valves based on a synthetic biodegradable PGA/polyester composite matrix was not affected by crimping, suggesting the feasibility of stented, catheter implantation. Although further study is required to assess the long-term function of these scaffolds in vivo, mounting evidence suggests that cell-free or minimally tissue engineered personalized valve scaffolds can provide immediate functional restoration with the potential for in vivo remodeling and improved host integration.

1.2.5 Conclusions for Engineering Fibrous Scaffolds

Heart valves are living tissues, capable of adapting its functional performance according to changing demands. By recapitulating the structure of the native heart using fibrous ECM-based scaffolds, it may be possible to build engineered heart tissues from self-organizing cells. Despite current limitations, we have begun to define the multi-scale, developmental, structural, and functional design criteria necessary to faithfully recapitulate the essential components of a working heart. Methods for mimicking the fibrous ECM such as decellularized tissue, electrospinning, and force spinning, provide the manufacturing methods and materials to build heart parts with relevant compositions and architectures for both in vitro and in vivo applications. In vitro platforms based on engineered cardiac tissues will increasingly be used to discover therapeutics and tandem approaches to implantable devices will facilitate host integration. However, incorporating bioactive components
within scaffolds to enhance their cell-instructive capabilities presents new challenges for safety and efficacy regulation. Diverse roles played by the ECM in regulating thrombosis, inflammation, angiogenesis, and mediating interaction between multiple cell types are largely unexplored in these systems. Although valve replacement with fibrous scaffolds may be achieved in the relative short term, the use of these scaffolds for myocardial repair requires further study \textit{in vitro} and \textit{in vivo}. Although routine clinical use of engineered scaffolds for cardiac tissue repair has yet to be achieved, our ability to re-create the heart from engineered component parts is continually advancing.

1.3 Graphical Approach

There remains a lack of early cardiac valve disease screening methods and permanent replacement strategies. Detailed in the thesis below, we have begun to address these needs following the approaches illustrated in Figure 1.7.

Figure 1.7: Approach to Improving Heart Valve Disease Diagnosis and Repair. We have developed an organ on chip model to identify early valve fibrosis using a drug-induced pathology case study and a rapid fiber production platform that enables the manufacture of functional, semilunar heart valves. (Healthy, Early Disease, and Chronic Fibrosis images from CTH Surgery)
2 In vitro Modeling of Drug-Induced Fibrosis in Valvular Interstitial Cell Tissues

Medications based on ergoline-derived dopamine and serotonin agonists are associated with off-target toxicities that include valvular heart disease (VHD). Reports of drug-induced VHD resulted in the withdrawal of appetite suppressants containing fenfluramine and phentermine from the U.S. market in 1997 and Pergolide, a Parkinson’s disease medication, in 2007. Recent evidence suggests that serotonin receptor activity affected by these medications modulates cardiac valve interstitial cell activation and subsequent valvular remodeling, which can lead to cardiac valve fibrosis and dysfunction similar to that seen in carcinoid heart disease. Failure to identify these risks prior to market, and continued use of similar drugs, reaffirms the need to improve preclinical evaluation of drug-induced VHD. Here, we present two complimentary assays to measure stiffness and contractile stresses generated by engineered valvular tissues in vitro. As a case study, we measured the effects of acute (24 hr) Pergolide exposure to engineered porcine aortic valve interstitial cell (AVIC) tissues. Pergolide exposure led to increased tissue stiffness but it decreased both basal and active contractile tone stresses generated by AVIC tissues. Pergolide exposure also disrupted AVIC tissue organization (i.e., tissue anisotropy), suggesting that the mechanical properties and contractile functionality of these tissues are governed by their ability to maintain their structure. From these data, we expect that the assays we present here can be further used to identify off-target drug effects that alter the phenotypic balance of AVICs, disrupt their ability to maintain mechanical homeostasis, and lead to VHD.
2.1 Introduction

A growing number of medications such as those used in the treatment of Parkinson’s disease and psychiatric disorders are dopamine/serotonin analogs or are designed to stimulate the production/reuptake of these neurotransmitters in the brain [178, 179]. Although both dopamine and serotonin are drug targets for neurological pharmacotherapies, their expression is ubiquitous throughout the body. In particular, they are highly expressed in the gastrointestinal and pulmonary tracts, as well as throughout the cardiovascular system, including the myocardium and cardiac valves [180, 181]. Consequently, chronic use of these drugs can cause significant patient morbidity via off-target, adverse effects such as pulmonary and cardiac fibrosis [182-185]. Ergoline-derived medications appear to be especially problematic as a number of anorectics [184] and Parkinson’s disease drugs [186] of this class cause fibrosis and gross structural changes to the cardiac valves, similar to the symptoms of carcinoid syndrome [184, 185]. These cases of drug-induced valvular heart disease are believed to result from excessive, chronic valvular interstitial cell (VIC) activation by serotonin 5HT-2B receptor agonism [187, 188]. Conversely, 5HT-2B antagonism inhibits myofibroblast activation of VICs in vitro, with increasing doses of 5HT-2B antagonist in the presence of transforming growth factor beta-1 (TGF-β1) leading to progressive decreases in alpha smooth muscle actin (α-SMA) expression after 24 hr [189]. Ergoline-derived medications therefore appear to be potent regulators of VIC activation and valve repair homeostasis but the time-course of action, progression to tissue-level mechanical dysregulation and potential reversibility are understudied. We reasoned that mechanisms by which VIC activation translates to VHD could be studied in vitro using
assays based on controlled VIC assembly into functional tissues that recapitulate key properties of natural valves.

VICs are a heterogeneous population of predominantly fibroblast-like cells found in all three layers of the valve leaflet extracellular matrix (ECM). VIC populations are rich in mesenchymal stem cells that have robust osteogenic calcification potential [190] and VIC activation from the quiescent fibroblast phenotype to a contractile myofibroblast phenotype (expressing α-SMA and SM22α [191]) confers reparative capacity to healthy valves [192, 193] and an ability to generate tissue tone [194]. VICs demonstrate a natural plasticity between these activated and quiescent phenotypes largely in response to their biochemical and mechanical environments. For example, TGF-β has been shown to cause dose-dependent activation of VICs and ECM remodeling [195, 196]. Mechanical properties of the valve niche that regulate VIC activation include transvalvular pressures, ECM stiffness, and fluidic shear forces [197-201]. Drugs can dysregulate the biochemical and mechanical homeostasis of VICs and cause chronic, pathological VIC activation that can lead to exaggerated matrix deposition, fibrosis, and calcification [184-187]. The eventual clinical manifestation of pathologically activated VICs presents as late-stage permanent valvular tissue dysfunction [202, 203]. In vitro assays aimed at identifying mechanisms of drug-induced VHD would benefit from tissue-level functional metrics that tie VIC activation to valve performance.

Acute, in vitro detection of pathological VIC differentiation has focused on mitogenic signaling pathways involved in excessive 5HT-2B receptor agonism, that include ERK1/2, Src, PKC, and/or TGF-β1 [187, 204-207]. These provide insight into potential treatment strategies but acute functional changes to valve tissues resulting from dysregulated
activation of these pathways remain largely unknown and undiagnosable clinically. We therefore asked whether VIC-based tissue stiffness and tissue tone stress generation were altered by acute exposure to 5HT-2B agonists. To answer this question, we engineered VIC tissues and measured changes in stiffness in response to exposure to 8β-methylthiomethyl-6-propylergoline (Pergolide), a potent 5HT-2B receptor agonist [208]. We then designed and built an in vitro VIC tissue tone stress assay to determine the effects of Pergolide on the tissue’s capacity to generate contractile stresses. By exposing engineered VIC tissues to a clinically relevant dose of Pergolide, we demonstrate that the characteristic tissue stiffening and disorganization of drug-induced valvulopathy is detectable after acute 24 hr drug exposure in vitro. Using our tissue tone assay, we demonstrate that acute Pergolide exposure reduced VIC tissue contractile capacity. Our results indicate that Pergolide-induced mechanical abnormalities that lead to clinically dysfunctional heart valve performance can be detected in vitro after acute exposures. These tissue-level, mechanical assays may provide a platform for drug screening and investigation into early stage drug-induced VHD.

2.2 Materials and Methods

We developed two distinct in vitro assays to measure VIC tissue stiffness and tone generation in response to acute Pergolide exposure. Although fibrotic carcinoid-like valvular pathologies occur primarily in the right heart valves (tricuspid and pulmonary) due to pulmonary clearance of excessive serotonin [209], Pergolide causes both left (aortic and mitral) and right (pulmonary and tricuspid) side valve dysfunction [183, 210-212]. Because the aortic valve fibrosa layer is most commonly associated with disease [125, 213], we engineered aortic VIC (AVIC) tissues that recapitulated aortic fibrosa ECM structure
(Figure 2.1a). For tissue stiffness measurements, we cultured AVICs on thin fibrous scaffolds that recapitulated the stiffness and structural anisotropy of healthy valve fibrosa tissues. Equibiaxial loading was used to measure the directionally dependent tensile elastic modulus of AVIC tissues exposed to Pergolide. To measure tissue tone stress regulation, we cultured anisotropic AVIC tissues on thin flexible cantilevers based on muscular thin film technologies previously developed in our laboratory [28, 214-217]. Cantilever bending radius, tracked optically, was proportional to tissue contractile force. Stiffness and contraction/tone assays were both performed using acute (24 hr) Pergolide exposures of 1 μM concentration.

2.2.1 Aortic Valve Interstitial Cell Source and Isolation

Primary AVICs were isolated from freshly harvested porcine hearts obtained in compliance with FDA guidelines (Blood Farms Inc., Groton, MA). Aortic valve leaflets were extracted from sacrificed hearts and kept in chilled phosphate buffered saline solution during transport. To harvest AVICs, intact leaflets were dissected from the whole heart and subjected to sequential collagenase digestions at 10 U/l in Hanks’ Balanced Salt Solution (HBSS, 55021C, Sigma Aldrich, St. Louis, MO) for 5 min to remove endothelial cells, followed by a 2.5 hr collagenase digestion at 37˚C to dislodge the AVICs from the tissue matrix. AVICs were filtered from the remaining leaflet matrix, centrifuged at 10,000 RPM for 10 min, and cultured in M199 media supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 20 mM glucose, 10 U/ml penicillin/streptomycin, 1.5 μM vitamin B12, 10 mM HEPES, and 0.1 mM non-essential amino acids. AVICs were cultured in T75 culture flasks coated with 1% porcine gelatin. At passage 2, AVICs were cryopreserved in liquid nitrogen.
Figure 2.1: Recapitulating the Leaflet Fibrosa with Biohybrid Rotary Jet Spinning.

Biohybrid scaffolds fabricated using the Rotary Jet Spinning (RJS) system were designed to mimic the circumferential alignment of the valve fibrosa extracellular matrix (ECM, a). The circumferentially oriented fibrosa “F” provides the mechanical strength of the leaflet while the spongiosa “S” is thought to provide cushioning during leaflet closure during diastole and the elastin-rich ventricularis “V” provides recoil after leaflet opening during systole (ai, leaflet cross-section). The inset to aii shows a scanning electron microscopy (SEM) image of decellularized porcine fibrosa (aii, SEM, scale bar 5 µm), which was recapitulated using RJS produced nanofibers. Circumferentially oriented nanofibers (bi, SEM, scale bar 20 µm) were seeded with AVICs and allowed 48 hrs of growth to form a confluent tissue (bii, SEM, scale bar 20 µm) followed by 24 hrs of Pergolide or no-drug exposure. After exposure, AVIC tissues were equibiaxially strained (biii, optical images, scale bar 5 mm) to determine drug induced changes in tissue level stiffness and strength (εi: initial strain at .05%, εf: final strain at 40%).
2.2.2 Engineered Aortic Valve Interstitial Cell Tissues

To recapitulate aortic valve leaflet structure \textit{in vitro} (Figure 2.1ai), we first fabricated fibrous polymer/bioprotein (“biohybrid”) tissue engineering scaffolds using Rotary Jet Spinning [48, 49, 218, 219]. Scaffold composition and fiber diameter specifications were formulated and sized based upon tissue engineered valve constructs previously reported in the literature [220-222]. Scaffold material precursors consisted of poly-4-hydroxybutyrate (55%, Tepha Inc., Lexington, MA), porcine gelatin (40%, G2500, Sigma-Aldrich), and polyglycolide (5%, 457620, Sigma-Aldrich, St. Louis, MO) homogenously mixed in hexafluoroisopropanol (003409, Oakwood Chemical, West Columbia, SC) at 4% w/v prior to fabrication. Fibers were extruded at 30,000 RPM and collected onto rotating cylindrical mandrels at 3,000 RPM to produce anisotropically aligned scaffold sheets approximately 200 $\mu$m thick; sheets were composed of fibers having an average diameter of approximately 1 $\mu$m (Figure 2.1bi). The composition and axial alignment of these scaffolds recapitulated the biaxial stiffness [223] and structural organization of the native valve leaflet ECM.

AVICs were seeded at a density of 200k cells/cm$^2$ onto 8x8 mm scaffold pieces cut with the squared faces parallel and perpendicular to the primary axis of fiber alignment (for all conditions and directions n=5 tissues). AVICs were then cultured for 48 hr in growth medium followed by a serum-free drug incubation period of 24 hr as detailed in Section 2.4 (Figure 2.1bii). After the drug incubation period, seeded scaffold pieces were individually loaded onto a biomaterials mechanical tester (BioTester, CellScale Inc., Waterloo, ON) and subjected to equibiaxial tensile loading with applied force ramped linearly between 0 and 2500 mN at a rate of 1 Hz (Figure 2.1biii). Force-displacement
measurements and corresponding images were recorded at 15 Hz. Tensile elastic modulus, E, was calculated using the slope of the tangent in the linear portion of the stress vs. strain curve and the ultimate tensile strength (UTS) was taken to be the largest stress value obtained prior to failure.

To visualize tissue formation, AVICs cultured on the biohybrid scaffolds were imaged using scanning electron microscopy (SEM). After the 24 hr drug incubation, tissues were fixed in 2.5% glutaraldehyde (G7776, Sigma-Aldrich) in 0.1 M HEPES (15630-080, Invitrogen, Carlsbad, CA) for 1 hr. Samples were then rinsed 3x in 0.1 M HEPES and 3x in D.I. water for 5 min each. Immediately following rinsing, samples were serially dehydrated in 30%, 50%, 70%, 90%, and 3x 100% ethanol (89125, VWR, Randor, PA) washes for 5 min each. Samples were dried for SEM imaging using a critical point drier (931 Series SAMDRI, Tousimis, Rockville, MD) and sputter coated in 5 nm of 80/20 platinum/palladium (EMS 300TD, Quorum Technologies, Lewes, East Sussex) to prevent charge accumulation and sample degradation during imaging. A field emitting electron microscope at 15 kV (FESEM Ultra Plus, Carl Zeiss, Oberkochen, Germany) with a high efficiency secondary electron detector was used to image samples.

2.2.3 Aortic Valve Interstitial Cell Tissue Tone Stress Assay

Based on muscular thin film (MTF) technology developed in our laboratory [28, 214-217], we engineered tissues of anisotropically patterned AVICs to produce contractile thin films. AVIC tissue alignment was engineered to recapitulate the native leaflet’s circumferential alignment in the fibrosa layer of the leaflet. PDMS thin films were fabricated using a spin coating and laser cutting processes previously reported by our laboratory [215]. Briefly, 22x22 mm glass coverslips (Product# 260300, Ted Pella Inc.
Redding CA) served as the substrate of the thin film chips and were covered in a low adhesion tape for masking (Product# 2080, 3M, St. Paul, MN). Two rectangular island shapes of dimensions 18x5 mm were cut from the tape with a CO\textsubscript{2} laser (Epilogue, Golden, CO) and removed with forceps (Figure 2.2ai). The thermosensitive polymer poly(N-isopropylacrylamide), PIPAAm, (Polysciences Inc., Warrington, PA) at 10% w/v in butanol was spin coated at 6000 RPM for 1 min onto masked coverslips with the rectangular islands removed (G3P8 Specialty Spin Coater, SCS Inc., Indianapolis, IN) to create PIPAAm islands (Figure 2.2a(ii)). Following PIPAAm spin coating, the masking was removed and polydimethylsiloxane, PDMS, (Slygard 184 elastomer, Dow Corning, Midland, MI) mixed at a 10:1 base to curing agent ratio was spin coated over the PIPAAm islands at 5000 RPM for 5 min to achieve a uniform 18 μm coating, (Figure 2.2aiii). Once cured, 1x3 mm cantilevers were cut into cured thin film chips over the PIPAAm islands with a CO\textsubscript{2} laser (Figure 2.2a(iv)).

To mimic the circumferential fibrosa alignment of the native valve, AVIC tissues were aligned in the direction of PDMS cantilevers using microcontact printing techniques. 20 μm lines of human fibronectin (BD Biosciences, Sparks, MD) separated by a gap of 20 μm were microcontact printed using PDMS stamps incubated for 1 hr with 50 μg/ml of fibronectin in D.I. water. After stamp incubation, chips were exposed to UV-ozone (Model# 342, Jetlight Company Inc., Phoenix, AZ) for 8 min then stamped with air-dried fibronectin coated stamps; stamps were brought in contact with chips for less than 1 min. Following fibronectin stamping, chips were immersed in a 5 μg/ml solution of fibronectin in D.I. water for 15 min to allow for a background coating of fibronectin between the stamped lines (Figure 2.2av).
**Figure 2.2: Fibrosa Contractile Thin Film Assay Fabrication.** The contractile thin film assay was designed to recapitulate the circumferential alignment of the fibrosa layer of the native leaflet ECM in 2D. The fibrosa is primarily composed of fibrous collagen bundles and cell-binding proteins such as fibronectin. To fabricate releasable thin films, a sacrificial layer of PIPAAM was spin coated into islands on a glass coverslip (ai: masked islands, aii: spin-coated PIPAAM within islands), followed spin coating a thin layer of PDMS (aiii) that was cured and laser-cut into cantilevers (aiv). AVIC tissues aligned in the direction of the cantilevers were created by microcontact printing 20x20 μm lines of fibronectin (av) and seeding AVICs onto the chips (avi). AVIC tissues were allowed a 48 hr growth period followed by a 24 hr Pergolide or no-drug exposure. Following drug exposure, cantilevers were released via temperature driven PIPAAM dissolution (b). bi shows a schematic of thin film release (left) and the insets (right) show confluent, aligned VICs (immuno-micrographs, scale bars 25 μm, blue: DAPI, black top: f-actin, black bottom: α-SMA). Thin film bending radius was tracked optically using a stereomicroscope and CCD (bii, optical images, scale bar is 10 cm; biii inset: thin film chip, scale bar is 5 cm).
AVICs were seeded onto stamped chips at a density of 100k cells/cm² (1M cells/thin film chip in a standard 6 well plate) and statically cultured for 48 hr in standard growth media detailed above in Section 2.2.1 (Figure 2.2avi, n=19 tissues for 1 μM Pergolide and n=18 tissues for no-drug). After 48 hr, AVIC tissues were rinsed in warmed phosphate buffered saline (PBS) at 37°C to remove potential residual serotonin, dopamine, or other potential confounding factors present in the media serum that may influence drug testing and cultured in serum-free media for 24 hr. During the 24 hr serum-free incubation, thin film chips were subjected to drug treatment of 1 μM Pergolide or no-drug (Pergolide: P8828, Sigma-Aldrich, St. Louis, MO). A 1 μM dose of Pergolide is a saturating concentration for stimulation of the 5HT-2B receptors of heart valve interstitial cells [224, 225] and is an approximation of the blood plasma concentration in the body of an adult patient taking 1-5 mg of Pergolide daily, a clinically relevant dosing range [183, 210].

After the 24 hr serum-free drug treatment, thin film chips were gently rinsed with and placed into 5 ml of Tyrode's solution at 37°C; all experiments were performed in 37°C warmed Tyrode’s solution (1.192 g/l HEPES, 0.040 g/l NaH₂PO₄, 0.901 g/l glucose, 0.265 g/l CaCl₂, 0.203 g/l MgCl₂, 0.403 g/l KCl, 7.889 g/l NaCl, pH adjusted to 7.4 using 1 N NaOH, all chemicals from Sigma-Aldrich, St. Louis, MO). To lift the PDMS cantilevers, chips in Tyrode's solution were allowed to briefly cool below 32°C, allowing PIPAAm to phase change into a hydrophilic state, thus releasing the cantilevers which were carefully peeled free from the PIPAAm islands with forceps (Figure 2.2bi). Once the cantilevers were released, the tissue tone assay was performed on a stereomicroscope (Figure 2.2bii, Model SteREO Discovery.v12, Zeiss).

During the assay, images were taken directly above the thin film chip (Figure 2.2biili)
every 30 sec. For the first 5 min of the assay, thin films were allowed to equilibrate and establish a basal tone tissue stress. Subsequently, AVIC tissue active contraction was induced via the known vasoconstrictor endothelin-1 (Et-1, E7764, Sigma-Aldrich, St. Louis, MO), at a 100 nM saturating concentration for 15 min. Next, cantilevers were completely relaxed with the addition of the rho-kinase inhibitor HA-1077 dihydrochloride (H139, Sigma-Aldrich) for 10 min at a 100 µM saturating concentration. Bending of the cantilevers as a result of basal tone and active contraction was detected using custom Image J (NIH, Bethesda, MD) software; radius of curvature and stress in the cantilever was then calculated using previously reported custom MATLAB code (Mathworks, Natick, MA) and models [28, 214-217].

2.2.4 Immunostaining and Tissue Alignment Analysis

PDMS-coated coverslips were microcontact printed with 20x20 µm fibronectin lines, seeded with AVICs, and cultured as described above (Section 2.2.3). Following drug incubation, coverslips were fixed in 4% paraformaldehyde (PFA, 15710, Electron Microscopy Sciences, Hatfield, PA) and 0.5% TritonX 100 (T8787, Sigma-Aldrich, St. Louis, MO) in PBS for 15 min. Coverslips were then rinsed 3x in PBS for 10 min each and incubated in 5% w/v bovine serum albumin (BSA, 001-000-162, Jackson ImmunoResearch, West Grove, PA) in PBS for nonspecific blocking. After the BSA block, coverslips were incubated in 200 µl of 0.5% w/v BSA solution in PBS containing 2 µl of monoclonal anti-alpha smooth muscle actin (mouse) and polyclonal anti-cofilin (rabbit) antibodies (ab7817 and ab11062 respectively; Abcam, Cambridge, Ma) for 2 hr. Following primary incubation, coverslips were rinsed 3x in PBS for 10 min each then incubated in 200 µl of 0.5% w/v BSA solution in PBS containing 5 µl of DAPI, 2 µl Alexa Fluor 633-conjugated phalloidin
(A22284, Invitrogen, Carlsbad, CA), 2 µl goat anti-mouse Alexa Fluor-488 (A-11001, Invitrogen, Carlsbad, CA), and 2 µl donkey anti-rabbit Alexa Fluor-546 (A-10040, Invitrogen, Carlsbad, CA). Coverslips were mounted on standard microscope slides and imaged using a Zeiss LSM 7 LIVE confocal microscope. All images were taken at 20x magnification producing a field of view of 160x160 µm; the number of nuclei within each field of view were used to determine tissue cell density. Fluorescent images of the actin cytoskeleton were used to calculate the Orientation Order Parameter (OOP), a relative measure of axial alignment in tissues [226, 227]. The OOP of a tissue is calculated based upon fingerprint identification algorithms which give a score of tissue anisotropy; an OOP score of 1 indicates perfect axial alignment while an OOP score of 0 indicates no preferential axis of alignment (n=8 tissues, 5 ROI/tissue for tissue cell density and OOP measurements; all reported as mean ± standard error of the mean).

2.2.5 Western Blot Protein Expression Analysis

To quantify changes in protein expression indicative of AVIC activation state, tissues microcontact printed and cultured as described above (Section 2.2.3), were lysed at 4°C in RIPA lysis buffer (SLBG8489, Sigma, St. Louis, MO) plus Complete Mini (11836153001, Roche Diagnostic, Mannheim, Germany) and Halt-Protease and Phosphotase Inhibitor (1861281, ThermoFisher Scientific, USA). Protein expression levels were quantified using a capillary-based Wes™ Simple Western Analysis (ProteinSimple, San Jose, CA). All procedures were performed according to the manufacturer’s protocol (cell lysates were pooled from n=5 tissues for each condition). Briefly, 1 µg cell lysates were loaded into each capillary and proteins were separated by size in the stacking and separation matrix. Target proteins, including α-SMA, cofillin, and α-tubulin (loading control), were identified
using primary antibodies (ab7817, ab11062, and ab7291, respectively, Abcam, Cambridge, MA). The proteins were immunoprobed using manufacturer provided secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified using Compass Software (Protein Simple, San Jose, CA).

2.2.6 Statistical Analyses

Changes in tissue stiffness, contractility, and alignment due to Pergolide exposure were statistically evaluated using SigmaPlot software (v12.0, Systat Software Inc., San Jose, CA). Tissue moduli and ultimate tensile strength in the parallel and perpendicular directions of scaffold fiber/tissue alignment for no-drug vs. 1 µM Pergolide (Section 2.2.2) were compared with t-tests, passing both the Shapiro-Wilk normality and equal variance tests. Active tissue tone stress generation for no-drug vs. 1 µM Pergolide (Section 2.2.3) failed the Shapiro-Wilk normality test (p<0.05) and thus were evaluated using the Mann-Whitney Rank Sum Test. Basal tissue tone stress generation for no-drug vs. 1 µM Pergolide (Section 2.2.3) passed Shapiro-Wilk normality and equal variance and were thus compared with t-tests. Changes in tissue alignment OOP for all no-drug vs. 1 µM Pergolide (Section 2.2.4) were evaluated with t-tests, passing both the Shapiro-Wilk normality and equal variance tests. For all statistical analyses, p-values less than 0.05 were considered statistically significant.
2.3 Results

2.3.1 Pergolide-induced Tissue Stiffening

The clinical manifestation of Pergolide-induced VHD is valvular dysfunction (e.g. regurgitation and/or stenosis) resulting from stiffened fibrotic leaflets [203]. We therefore asked if Pergolide-induced AVIC tissue stiffening could be measured *in vitro* using biaxial tensile testing of engineered AVIC tissues. AVIC tissues were engineered on valve-like scaffolds designed to mimic the fibrosa layer of the leaflet and exposed to 1 µM Pergolide for 24 hr following a 48 hr period of tissue formation (Figure 2.3a). After exposure, tissues were equibiaxially strained and force-displacement data were used to measure Pergolide-induced changes in tissue tensile modulus, E, and UTS (Figure 2.3b). E values measured in the primary axis of fiber/tissue alignment (parallel) increased by 27% following 24 hr exposure to 1 µM Pergolide (t-test p<.05, Figure 2.3ci). E values in the perpendicular axis of fiber/tissue alignment increased by 23% following 24 hr exposure to 1 µM Pergolide (t-test p=0.49, Figure 2.3cii). No differences in UTS were observed. These data suggest a directionally dependent increase in tensile modulus (stiffness) along the axis of tissue alignment resulting from acute (24 hr) Pergolide exposure.

2.3.2 Reduction of Tissue Tone Generation and Structural Anisotropy

The increased tensile modulus that we observed in AVIC tissues exposed to an acute Pergolide dose suggests that Pergolide induced a phenotypic change towards synthetic AVIC tissues. We therefore asked if a change from the fibroblast-to-myofibroblast phenotypic activation state in AVIC tissues was induced in response to acute Pergolide exposure. Immunostaining and protein quantification of AVIC activation
markers revealed expression of both α-SMA and coflin in both no-drug and Pergolide treated tissues. α-SMA staining in no-drug AVIC tissues appeared more continuous and uniform throughout the tissue when compared to Pergolide treated tissues. Cofilin staining in no-drug AVIC tissues appeared more diffuse compared to Pergolide treated tissues (Figure 2.4ai), where coflin revealed a high degree of co-localization with cytoskeletal f-actin (Figure 2.4a(ii)). Western blot protein expression showed a 30% decrease in α-SMA expression in Pergolide treated AVIC tissues, whereas coflin expression was unchanged by drug exposure (Figure 2.4b). Given that AVIC activation has been shown to decrease...
with increased cell density [228], our data taken from confluent tissues suggest that Pergolide exposure increases this contact driven activation-to-quiescence mechanism acutely in AVICs.

Figure 2.4: Pergolide Induced Tissue Stiffening on Fibrous Scaffolds. No-drug and Pergolide treated AVIC tissues expressed both alpha smooth muscle actin (α-SMA) and cofilin. No-drug AVIC tissues exhibited more uniform α-SMA staining throughout the tissue (ai, red) while cofilin staining was more diffuse within cells (ai, green). 1 μM Pergolide treated tissues exhibited less pronounced α-SMA staining throughout tissues (a(ii, red)) but a higher degree of cofilin-actin colocalization (a(ii, white: f-actin and green: cofilin) than no-drug tissues (scale bar 50 μm for all images; n=3 tissues per condition). Protein quantification revealed a 30% relative decrease in α-SMA expression due to acute Pergolide exposure while no difference was observed in relative cofilin expression due to the drug (b, cell lysates were pooled from n=5 tissues for each condition, proteins of interest normalized to α-tubulin as the loading control).

We then asked if this phenotypic shift would affect the AVIC tissue’s ability to modulate tissue tone. To measure tissue tone in vitro, anisotropic AVIC tissues were
engineered using microcontact printing on thin film cantilever substrates; after 48 hr of growth, tissues were exposed to 1 µM Pergolide for 24 hr. Following exposure, thin film tissues were released at one end permitting optical measurement of active and basal tissue tone stress generation (Figure 5A). Released cantilevers were given 5 min to acclimate and reach their basal tone (Figure 5Bi) followed by 15 min of Et-1 induced active contraction (Figure 5Bii) and 10 min of HA-1077 induced relaxation (Figure 5Biii). Although AVIC tissues in both no-drug and 1 µM Pergolide treated groups were equally dense (Figure 5Ci), Pergolide exposed tissues had a significantly reduced active and basal tone stress generation (t-test p<.05, Figure 5Cii active and 5Ciii basal). Because of the unidirectional nature of our contractility assay, we asked if these observed losses in active and basal tissue tone were influenced by changes in tissue anisotropy, i.e. deviation in alignment relative to the contractile direction of the thin films. As measured by their f-actin OOP, 1 µM Pergolide treated tissues had a reduction in tissue alignment of 7% relative to no-drug controls (t-test p<.05, Figure 5D). Taken together, these data suggest that acute Pergolide exposure reduces AVIC tissue stress generation capacity, which is due, at least in part, to a loss of axial tissue alignment.
Figure 2.5: Pergolide Induced Loss of Tissue Tone Generation and Anisotropy. Following AVIC tissue growth, exposure, and thin film release the tissue tone generation assays were conducted (a). First, a basal tone was established for 5 min (bi) followed by the induction of active stress generation via administration of 100 nM endothelin-1 (Et-1, bii). Following 15 min of active contraction, 100 µM HA1077 was administered to induce full relaxation of the cantilevers for 10 min (biii; scale bars in b 1 mm, all optical images). No differences in tissue cell density were observed among the conditions (ci, n=4 tissues with 5 FOV/tissue for no-drug tissues and n=5 tissues with 5 FOV/tissue for Pergolide treated tissues). Tissues exposed to 1 µM Pergolide exhibited reduced tissue tone generation of 7.60 ± 1.19 kPa active tone (cii, red) and 4.28 ± 0.80 kPa basal tone (ciii, red) compared to no-drug controls which exhibited tissue tone generation of 13.40 ± 2.23 kPa active tone (cii, blue) and 6.97 ± 1.37 kPa basal tone (ciii, blue). All conditions reported as mean ± standard error of the mean (n=19 tissues for 1 µM Pergolide and n=18 tissues for no-drug, *p < 0.05). To investigate tissue alignment, OOP of the cytoskeleton were measured (d, immuno-micrographs, scale bars 50 µm; blue: DAPI, black: f-actin). Tissues exposed to 1 µM Pergolide exhibited a decreased OOP of 0.82 ± 0.02 (d, n=9 tissues, 5 ROI/tissue) while no-drug tissues exhibited an OOP of 0.88 ± 0.02 (d, n=8 tissues, 5 ROI/tissue; all reported as mean ± standard error of the mean, *p < 0.05).
2.4 Discussion

Using engineered AVIC tissues exposed to an acute Pergolide dose, we observed a significant increase in tissue tensile modulus along the primary axis of fiber/tissue alignment. This increase in tensile modulus of our thin AVIC tissue model is suggestive of the drastic increases in stiffness observed clinically in cases of valve fibrosis. Fibrotic valve lesions observed in patients pathologically affected by Pergolide are accompanied by leaflet tissue stiffening that ultimately leads to valvular insufficiency [186, 229]. The current standard of care for monitoring drug-induced functional heart valve irregularity is echocardiography, usually performed at roughly 6 month intervals [230]. Our results indicate that pathological changes in biaxial valve tissue stiffness can potentially be observed and quantitatively measured on the order of days rather than months. Future studies may determine whether early onset stiffness changes can be observed clinically using high resolution echocardiography [231] or MRI-based [232] non-invasive imaging techniques.

Our results support the hypothesis that tissue-level Pergolide-induced pathologies may, at least in part, be due to a shift in AVIC phenotype towards an overly synthetic activation state [233]. This was further evident in the effect of Pergolide on our contractile thin film assay; that is, stiffer and more synthetic AVIC tissues were not able to generate as much tissue tone. When treated with the same Pergolide dose (1 μM), the thin film tissue stress assay showed a nearly 50% reduction in both active and basal tone stress generation. The significantly reduced contractility following drug exposure is further suggestive of the potential off-target effect of Pergolide on the cardiac valves. In particular, as AVICs lose their stress generation capacity, they may lose their ability to maintain tissue tone within
the leaflet, thus disrupting their homeostatic mechanical environment. A loss in this mechanical feedback mechanism may result in further and more permanent AVIC pathological activation within the tissue [234].

α-SMA and cofilin staining and expression quantification were consistent with the observed AVIC tissue tone stress generation data. The expression of α-SMA, a protein critical to the contractile mechanism of AVICs, was decreased in Pergolide treated tissues, which is consistent with the observed decrease in active and basal stress generation relative to no-drug treated tissues. Additionally, increased cofilin-actin colocalization in Pergolide treated tissues suggests increased cell motility within the tissue [235] which is an indicator of early myofibroblast response to injury [236]. Colocalization of cofilin with cytoskeletal f-actin is necessary for eventual stress fiber assembly [237] and has been proposed to be required for cardiac myofibroblast differentiation after force-induced injury of the myocardium [238]. As a result, the initial decrease in α-SMA expression and increase in actin-colocalization of cofilin in Pergolide treated AVIC tissues observed here may reflect early “proto-myofibroblast” stages of permanent myofibroblast differentiation in our engineered tissues.

Additionally, the loss of functional tissue tone generation in our contractility assay is indicative of a loss in the AVIC population’s stress generation capacity in a single direction. Because our AVIC tissues were engineered to be initially aligned in the direction of the thin film cantilevers, a loss in tissue alignment will reduce measured contractile stresses. We observed a 7% loss of AVIC alignment in response to 1 µM Pergolide exposure, as quantified using the orientation order parameter. The Pergolide-induced reduction in measured AVIC tissue contractility therefore likely resulted from increased tissue stiffness,
early reduction in α-SMA expression, and loss of tissue alignment. This is in agreement with previously reported mathematical models of varied tissue orientation and thin film mechanics [239] as well as drug-induced changes in tissue alignment that influenced smooth muscle cell stress generation [240].

The standard of any in vitro drug screening platform or organ-on-a-chip technology [29, 241] is its relevance to the clinic. In the case of this study, the retrospective and pathological comparisons of Pergolide-induced VHD observed clinically and those observed using our platforms must be critically compared. We aimed to expand upon the current mechanistic study of drug-induced VIC pathology by developing tissue-scale mechanical models. The AVIC tissue assays we built showed pronounced functional changes in AVIC tissue tone generation, loss of tissue alignment, and a significant increase in tissue tensile modulus following acute drug exposure. In particular, the tissue modulus and alignment results draw direct comparison to the gross structural and mechanical changes of Pergolide-induced VHD observed clinically and in explanted tissue [242]. At the cellular-scale, markers of off-target cardiac valve pathologies induced by Pergolide and other ergoline-derived medications may include morphological changes in tissue alignment or other biological markers such increased chronic α-SMA expression and excessive mitogenic activity [243]. However, we additionally propose that an increase in stiffness and loss of tone generation capacity at the tissue-scale may serve as effective functional markers of early drug-induced VHD for current and future neurological medication evaluation and development. Many non-specific dopamine agonists including the drugs 3,4-methylenedioxymethamphetamine (MDMA) [224], fenfluramine-phentermine [184, 244], and cabergoline and Pergolide [186, 202, 245] have been shown to cause valvular
dysfunction. Similar new dietary, Parkinson’s disease, and other psychiatric drug
development designed to act on serotonin and/or dopamine may benefit from these
platforms as techniques to determine early warning signs of VHD. The *in vitro* assays
presented here are well-suited to study AVIC activation and tissue-level consequences of
AVIC-myofibroblast dysregulation and differentiation. We expect further use of these
assays to identify off-target drug effects that alter the phenotypic balance of AVICs, disrupt
their ability to maintain mechanical homeostasis, and lead to VHD [187, 192, 193, 246].

2.5 Conclusion

We designed and built two complimentary *in vitro* assays to measure AVIC tissue
stiffness and contractile capacity, and validated their use for preclinical drug screening
using a known valvulopathogen, Pergolide. Our contractile thin film tissue tone assay was
sufficiently sensitive to reveal Pergolide-induced reduction of AVIC tissue contractile tone
concomitant with increased tissue stiffness and loss of tissue anisotropy measured
independently. These results highlight the role of AVICs in the maintenance of tissue tone,
are suggestive of the acute off-target effects of Pergolide and, potentially, other 5HT-2B
receptor modulators. Because our assays are based on measurements of tissue-level
pathologies, they will serve as effective preclinical drug screening assays that bridge cell-
based assays with clinical evaluation methods.

**Study Limitations:** This study was limited to acute, 24 hr, drug exposure; therefore,
these data are indicative of the very early onset of off-target drug effects on the cardiac
valves, of which we have little direct clinical comparison. Therefore, these results are
suggestive of the chronic pathologies observed clinically and further time points are
required to investigate the full etiology of ergoline-derived drug-induced VHD.
Additionally, all samples were cultured under static conditions thus not mimicking the pulsatile flows and pressures that the native valve is exposed to. The native valve is largely composed of VICs covered by a monolayer of valvular endothelial cells (VECs) that regulate VIC phenotype [247]. Although we chose to study AVICs in isolation, future studies will include VIC/VEC co-cultures.
3 Fibrous Scaffold Manufacturing of Functional Semilunar Heart Valves for Regenerative Therapies

Tissue engineered scaffolds have emerged as a potential solution for heart valve replacement because of their capacity for regeneration. However, traditional heart valve tissue engineering has relied on resource-intensive, cell-based manufacturing, which increases cost and hinders clinical applications. Alternatively, in situ tissue engineering approaches provide a potential solution based on scaffold replacements that elicit endogenous tissue remodeling and repair. Yet despite recent advances in synthetic materials manufacturing, there remains a lack of cell-free, automated approaches for rapidly producing biomimetic heart valve scaffolds. Here, we designed a jet spinning process for the rapid and automated fabrication of extracellular matrix mimicking heart valve scaffolds compatible with minimally-invasive transcatheater implantation strategies. We were able to fabricate customized biomimetic scaffolds in minutes, controlling the multiscale architecture, mechanical properties, and biochemistry of the resorbable scaffold fibers. We also tested scaffold biocompatibility and functionality in vitro and in vivo as a pulmonary valve replacement in an acute ovine model demonstrating performance comparable to the native valve.

3.1 Introduction

Historically, heart valve tissue engineering has relied on cell-based manufacturing to build living tissues in vitro [248]. In this approach, valvular progenitor cells are seeded onto scaffolds and conditioned in bioreactors that mimic the physiological conditions (pressures and flows) of the native valve. The conditioned cells remodel the scaffold in order to produce a microenvironment that mimics the complex spatial organization,
mechanical properties, and biochemical composition of the native leaflet extracellular matrix (ECM) [249]. These conditioned scaffolds thus mimic the native ECM to mediate the inflammatory response upon implantation and provide a microenvironment that supports tissue repair and regeneration [250]. In recent attempts to improve translation of tissue engineered valvular scaffolds, storage has been made possible by decellularizing [222, 251] conditioned scaffolds, which can be recellularized prior to [252] or after implantation [253]. Although this strategy has shown promise as both a functional and regenerative approach, these “off-the-shelf” valvular scaffolds can take months and cost thousands of dollars to produce using manual and non-standardized manufacturing techniques. Fabrication steps including cell sourcing/isolation [254] and cell/scaffold conditioning [255, 256] in heavily regulated GMP environments are complex and may require the patient to take immunosuppressive therapies if foreign biologics [10] or non-degradable materials are used. As a result of the manufacturing time, cost, and inherent potential for product variability [257], the translation of tissue engineered heart valves to the clinic remains limited [258, 259].

In situ heart valve tissue engineering is a simplified method for permanent, regenerative valve replacement [260]. In this approach, the scaffold is designed to promote endogenous mechanisms that drive tissue formation and remodeling once implanted [253, 259]. The manufacturing process must therefore be capable of producing scaffolds that both modulate the direction of blood flow in the heart immediately upon implantation and recapitulate the microenvironment of the native valve ECM to promote endogenous remodeling [261]. To achieve this, numerous materials fabrication techniques such as fiber electrospinning [262] and force spinning [48], hydrogel molding [263], and 3D/bioprinting
[264] have been developed to fabricate biomimetic, regenerative valvular scaffolds, each with unique building advantages. The nanoscale resolution of fiber production systems, the simplicity of hydrogel material properties manipulation, and the customizable global designs achievable with 3D printing each recapitulate central aspects of valvular architecture. However, new manufacturing techniques achieving both high resolution and degree of customization remain needed to mimic 1) the anisotropic architecture of the valvular ECM, 2) the stiffness of the leaflet to withstand systolic and diastolic loading, and 3) incorporate native ECM proteins to allow for cellular attachment and initiation of the remodeling process [11, 256].

In this study, we introduce a cell-free manufacturing technique for the rapid production of biomimetic semilunar heart valve scaffolds (JetValves). JetValves were manufactured in a two-step mandrel collection process which enabled facile shape and size customization. By varying the biohybrid composition of JetValve scaffolds, the multiscale architecture, mechanical properties and biochemistry of the scaffold fibers were engineered to recapitulate those of the native valve ECM (ovine). The controlled and automated fabrication of JetValves enabled seamless and rapid production (minutes from raw material to product) allowing for the implementation of basic quality control standards to ensure scaffold consistency prior to use. JetValves demonstrated functionality in vitro and biocompatibility/competency in vivo upon minimally invasive implantation into an ovine pulmonary valve model. The JetValve fabrication process provides a versatile platform for the rapid production of in situ tissue engineered heart valve scaffold design and manufacture.
3.2 Materials and Methods

3.2.1 JetValve Mandrel-Based Manufacturing Process

Tri-leaflet valvular scaffolds (JetValves) were three-dimensionally manufactured by the cumulative collection of force-extruded fibers onto custom sized mandrels. A two-step collection process was used via automation of the Rotary Jet Spinning system (aRJS, Figure 3.1a) [48, 49] controlled by a customized LabVIEW interface. First, the leaflet cusps were spun by the collection of fibers onto a rotating semilunar valve leaflet shaped mandrel (male-mandrel) cyclically translating through the fiber extrusion plane. By the subsequent addition of a shielding mandrel (female-mandrel) over the leaflet mandrel and further fiber deposition, the leaflets were seamlessly spun into a fibrous conduit to produce the semilunar valve within a vessel structure (Figure 3.1b). Once dried, removal of Teflon or 3D printed, Teflon-coated collection mandrels from either end of the fibrous conduit was possible without disrupting its structure.

3.2.2 JetValve Fabrication for Structural and Functional Testing

Poly-4-hydroxybutyrate (P4HB, Tepha Inc., TephaFLEX)/gelatin (Sigma, G2500) “biohybrid” [49] (synthetic polymer/protein) solutions of varied composition (P4HB/gelatin; 100/0, 80/20, 60/40, 40/60, and 20/80) were stirred for 12 hr at 4% w/v in hexfluoroisopropanol (HFIP, Oakwood Chemical, 003409) to ensure homogenous mixing. A total of 0.2% w/v of polyglycolide (Sigma, 457620) was added to each solution for compositional comparison to previously published valvular scaffolds [222, 251, 252]. After mixing, solutions were individually pumped into the rotating reservoir of the aRJS at 5.0 ml/min through polyfluoroalkoxy alkane tubing (Saint-Gobain, TSPF35-0125-031-50) using an automated syringe pump (Harvard Apparatus, 703007). The reservoir of the
Figure 3.1: Automated Rotary Jet Spinning of JetValve Manufacturing. To reproducibly and rapidly recapitulate native leaflet fibrosal structural alignment in JetValve scaffolds, the collection of extruded fibers from the Rotary Jet Spinning system was automated (aRJS, a). Biohybrid (synthetic polymer/protein) solutions were extruded from the aRJS reservoir, solidified into fibers within the fiber extrusion plane via solvent evaporation, and collected onto semilunar valve shaped mandrels to three dimensionally produce the JetValve scaffold. The leaflet structure was first spun onto the leaflet mandrel (1, male) onto which the shielding mandrel (2, female) was subsequently fixed and again spun to form the three dimensional structure of a semilunar valve vessel (b). Mandrels were machined from Teflon to allow for facile removal of male and female portions from either end of the construct to form the macro structure of the valve; that is, seamlessly formed semilunar leaflets within a conduit as depicted by the cross sectional view.
aRJS was machined from stainless steel to ensure structural stability at high speeds and rotated at 30k RPM (motor: Nakanishi, EM-3080J) to extrude solution jets within a horizontal “fiber extrusion plane.” Solution jets were extruded from two 360 µm diameter orifices drilled horizontally into the bottom of the reservoir with a #80 drill bit. Fibers ranging from nano to micron scale diameters, depending on the P4HB/gelatin solution ratio, were formed and collected onto semilunar valve shaped rotating mandrels as described above. Mandrels were rotated at 3k RPM at 45º relative to the horizontal as they vertically translated through the horizontal fiber extrusion plane at 10 cm/s (linear motor: Misumi, LX20) to collect fibers. Mandrel rotation and vertical translation was automatically controlled within a custom LabVIEW user interface (National Instruments, v12.0.1f4). JetValve scaffolds manufactured for ovine implantation were 30 mm in diameter and composed of 60/40 P4HB/gelatin; 30 ml of solution were spun to form the leaflets and 40 ml were spun to form the remainder of the housing, vessel conduit. 8 mm wide sample strips were cut from either end of each JetValve scaffold for quality control testing.

3.2.3 Mandrel Scaling and Customization

JetValve mandrels were custom-drawn and scaled using computer aided design software (Solidworks, 2015) and milled from Teflon for implantation (Proto Labs) or 3D printed in Rigid Opaque photopolymer for rapid scaling (Blue, Stratasys, Object30 Printer). Leaflet and shielding mandrels were scaled from 30 mm (ovine, implantation model used in this study) to 750 um in diameter (mouse) to produce JetValves of various sizes (Figure 3.2a). Furthermore, sinuses were added to the shielding mandrel to produce aortic valve relevant geometries using the same, two-step mandrel spinning method of fabrication described above. A “Sinus Cylinder” for housing “Sinus Cartridges” comprised the
shielding-female mandrel and allowed for seamless sinus bulge incorporation into the JetValve with easy mandrel removal via breakdown of the cylinder-cartridge assembly once dried (Figure 3.2b).

**Figure 3.2: Mandrel Scaling and Aortic Sinus Inclusion.** By 3D printing mandrels, the JetValve fabrication technique was made amenable to animal model and ultimately patient-specific sizing and customization of valvular features (a). Eventual aortic applications of JetValve scaffolds will require the inclusion of sinus bulges while maintaining the seamless leaflet-conduit interface (b). To accomplish this, the female-shielding mandrel was redesigned into individual, symmetric sinus component “cartridges” which, when taken apart from the sinus “cylinder”, allow for non-disruptive removal from the conduit, maintaining the seamless three dimensional architecture of the aortic JetValve scaffold.
3.2.4 Fiber Diameter and Scaffold Porosity

Scanning electron microscopy (SEM) and ImageJ software (NIH, v1.48s) were used to measure the fiber diameter and percentage porosity of JetValve scaffolds of varied biohybrid composition (P4HB/gelatin; 100/0, 80/20, 60/40, 40/60, and 20/80). The sample size per scaffold was n=6 samples, cut with a 4 mm biopsy punch from spun sheets or, in the case of implanted JetValves, the 8 mm sample strip after manufacture; three of the samples were mounted flat for fiber diameter measurements and three were mounted in cross-section for porosity measurements. Samples were sputter coated in 5 nm of platinum/palladium (Quorum Technologies, EMS 300TD) to avoid charge accumulation and scaffold deformation/melting during SEM imaging. A field emitting electron microscope was used to image samples (Zeiss, FESEM Ultra Plus) at 15 kV with a high efficiency secondary electron detector, 1.75k magnification for fiber diameter and 1k magnification for porosity images. Using ImageJ, 10 fiber diameter regions of interest (ROIs) were taken per image (30 per scaffold) using the linear measuring tool while one porosity ROI was taken per image (3 per scaffold) using thresholding percentage (percent porosity: fiber vs. non-fiber in cross-section).

3.2.5 JetValve Leaflet Fiber Orientation

The fiber orientation of 60/40 P4HB/gelatin JetValve scaffold samples (n=16 JetValve leaflet bellies) was compared to that of decellularized ovine pulmonary valve leaflets (n=7 ovine leaflet bellies). Freshly harvested pulmonary leaflets were decellularized in 1% sodium dodecyl sulfate (SDS, Sigma-Aldrich, L6026) for 4 days. After decellularization, leaflets were rinsed in ultra-pure water (Thermo Fisher, 10977-015) then dehydrated in serial ethanol washes (30%, 50%, 70%, 90%, 3x 100%) for 5 min each.
Dehydrated leaflets were then dried using a critical point drier (Tousimis, 931 Series SAMDRI) and sputter coated; SEM images of scaffolds and decellularized tissue were taken as described above. SEM images were analyzed with custom made ImageJ and Matlab software to calculate the orientational order parameter (OOP), a quantitative measure of the degree of fiber orientation within a scaffold/tissue. In brief, foreground pixels were assigned the orientation of the local neighborhood using a structure tensor method; then, the set of all orientations was summed assuming they represented the directions of vectors of unit magnitude. The result is a number that goes from 0, for perfectly random set of orientations, to 1 for perfectly aligned foreground pixels [103, 265].

### 3.2.6 Scaffold and Tissue Biaxial Mechanical Properties

JetValve scaffolds of varied biohybrid composition (P4HB/gelatin; 100/0, 80/20, 60/40, 40/60, and 20/80) were equibiaxially loaded to determine low (0-10%) and high (10-20%) strain stiffness measurements in comparison to freshly harvested ovine pulmonary leaflets. 8x8 mm scaffold samples (n=6 sheets per biohybrid condition) and 8x8 mm native leaflet samples (n=5, cut from cusp centers) were loaded onto 5x5 mm mounting tines of a biaxial tensile tester equipped with 2.5 N load cells (CellScale, BioTester) [266]. Loaded samples were submerged in a phosphate buffer saline (PBS, Thermo Fisher, 10010023) bath at 37ºC to simulate hydration and temperature conditions in vivo; all tests were run in the warmed PBS bath. Each sample was first preconditioned equibiaxially at a strain rate of 5% per second to 2% strain 4 times to ensure complete hydration of the scaffold or tissue. Next, each sample was loaded equibiaxially at a strain rate of 5% per second to 20% strain 4 times. Force/displacement measurements and images were recorded
throughout the test at 15 Hz; stress vs. strain plots were then generated from these measurements and the original dimensions of the samples (i.e. thickness and length in each direction, parallel and perpendicular to fiber/ECM orientation/direction). Stiffness moduli were calculated as the slope to the stress vs. strain curves in the respective low and high strain regimes.

3.2.7 Scaffold Composition Measurements

Fourier Transform Infrared Spectroscopy (FTIR) was used to measure the relative polymer crystallinity and protein content in JetValves. Initial relative protein content within JetValves was measured by comparing the height of the carbonyl stretch peak (1720 cm\(^{-1}\), indicative of the polyesters polymer) with those of the amide I and amide II peaks (1645 and 1535 cm\(^{-1}\) respectively, indicative of gelatin). As an initial measure of biohybrid protein retention in an aqueous environment, the spectra of aRJS versus electrospun fibers were evaluated for up to 7 days in ultra-pure water at 37°C and relative peak heights compared for content and homogeneity.

X-ray photoelectron spectrometry (XPS, Thermo Scientific, K-Alpha XPS,) was used to evaluate freshly-spun and hydrated JetValve scaffold composition in time. 8x8 mm pieces of 60/40 P4HB/gelatin scaffolds were hydrated in 1 L of ultra-pure water and stored in an incubator at 37°C for up to 7 days. Hydrated samples were removed from the water bath every 24 hr with sterile forceps and were dried for 12 hr under vacuum, after which their composition was evaluated using the XPS system (n=3 scaffold pieces and XPS measurements per time point, 0-7 days). Briefly, each sample was etched for 30 seconds at 500 eV (medium) to remove any surface debris that may have accumulated during sample preparation and was survey scanned over a 400 μm\(^2\) spot size. Gelatin ratiometric content
was estimated based upon the measured presence of nitrogen in the sample and the amount of bound solvent (HFIP) residual was estimated based upon the measured presence of fluorine, each normalized to the element's percentage within the respective molecule. Under the assumption that sample purity was maintained throughout the hydration, drying, and etching process, P4HB/PGA content was estimated as the remaining scaffold percentage non-gelatin or solvent.

3.2.8 Scaffold Batch Process Capability

The batch process capability [267] (±3 times the variance) was used to evaluate the manufacturing accuracy and precision of aRJS produced 60/40 P4HB/gelatin JetValves for implantation. For fiber diameter, the upper control limit (UCL) was set at 1.2 μm and the lower control limit (LCL) was set at 0.8 μm based upon previously published tissue engineered scaffolds [222, 251, 252]. As scaffold porosity is a function of fiber diameter using the aRJS manufacturing technique and JetValve input materials, the UCL was set at 50% and the LCL was set at 30% given the achievable range and reproducibility of the manufacturing process. All stiffness measurement control limits were based upon the measured stiffnesses of freshly harvested pulmonary leaflets: low strain stiffness in the parallel fiber direction control limits ranged from 0-10 MPa, low strain stiffness in the perpendicular fiber direction control limits ranged from 0-1 MPa, and high strain stiffness in the parallel and perpendicular fiber directions control limits ranged from 0-20 MPa. For scaffold thickness, sample strip thicknesses ULC was set at 600 μm and the LCL was set at 250 μm to ensure scaffolds were thin enough to be crimped consistently but not too thin as to lose structure once crimped and/or loaded in vivo. Protein content was taken as the relative amount of polyester to gelatin in the scaffold as measured by comparing the peak
height of the carbonyl stretch peak at 1720 cm\(^{-1}\) (indicative of P4HB/PGA) to the amide I peak at 1645 cm\(^{-1}\) (indicative of gelatin) using Fourier Transform Infrared Spectroscopy (FTIR) [49]; the protein content UCL was set at 2.5 and the LCL set at 0.5. The scaffold fiber orientation was measured using the OOP based upon the OOP measured in decellularized native tissue as described above; the OOP UCL was set at 0.8 and the LCL was set at 0.375.

3.2.9  **In vitro Functional Testing**

Preliminary evaluation of JetValve functionality using an *in vitro* pulse duplicator system (Vivitro Labs, Pulse Duplicator) was conducted to ensure the integrity of the scaffold leaflet design under physiologic pressures and flows [268]. 60/40 JetValves were anchored into 30 mm diameter nitinol stents with 5-0 suture (Ethicon, black monofilament) and continuously loaded for 48 hr under pulmonary conditions. A FDA waveform at 70 beats per minute was applied to the pulse duplicator’s 150 ml silicon ventricle; distal compliance chambers were adjusted to achieve pulmonary-like pressures across the JetValve during diastole. Ventricular (proximal to the JetValve) and arterial (distal to the JetValve) pressures and intravalvular volumetric flow rates were measured at 48 hr at a sampling rate of 256 samples per cycle.

3.2.10  **In vivo Implantation Deployment and Functional Testing**

60/40 JetValves were implanted into the orthotopic pulmonary valve position of ovine models for deployment and acute functional testing via transapical access (Figure 3.3a), a delivery technique recently shown to be a viable implantation method for tissue engineered heart valves [222]. The ethics committee (ZH151_2013, Zürich, Switzerland) approved the study in compliance with the Guide for the Care and Use of Laboratory
After a right sided thoracotomy, the pericardium was opened and the right ventricle (RV) was exposed, before it was punctured using needle through purse-string sutures. Next, a guide wire was introduced into the RV and placed into the main pulmonary artery under fluoroscopic control. The scaffold loaded implantation catheter was introduced into the RV over the wire and placed over the native pulmonary valve. Optimal positioning was controlled by contrast angiography, before the scaffold was delivered under fluoroscopic control. After full delivery of the scaffold a final contrast angiography was done to ensure optimal positioning, instant functionality and complete exclusion of the native pulmonary valve. The delivery device was removed, the RV was closed with the purse-string, and the thoracotomy was closed. Finally trans-esophageal echocardiographic assessment was performed postoperatively and at 15 hr to evaluate valve functionality (n=4 JetValve implants into the pulmonary position of separate animals).

For deployment, JetValves were crimped from 30 mm in diameter to 9 mm in diameter and loaded into the implantation catheter. Stress minimization during crimping was a critical design concern because of the non-woven micro-structure of the scaffold and resulting potential susceptibility to suture and shear-induced tearing. To test capacity for crimping, JetValves were anchored into nitinol stents via suture and pneumatically crimped from 30 mm (fully extended, adult ovine size) to 9 mm (fully crimped) at 45 psi. Leaflet shape and thickness (~300μm) were optimized to ensure a “swirling” fold during the crimping process that minimized the stresses on the leaflets and leaflet-conduit sutured anchor points (Figure 3.3b).
For scaffold fiber diameter, scaffold porosity, and scaffold stiffness measurements, One Way Analyses for Variance between the biohybrid compositional groups were conducted using SigmaPlot (v12.0, Systat Software Inc.). For fiber diameter, Shapiro-Wilk normality failed, thus a Kruskal-Willis One Way Analysis of Variance Ranks Test was conducted; for pairwise multiple comparison among scaffold groups, a Tukey Test was conducted (diameter: \( n=6 \) samples per biohybrid condition, 10 FOV per scaffold). Scaffold porosity passed Shapiro-Wilk normality (\( P=0.954 \)) and equal variance (\( P=0.680 \)); for

**Figure 3.3: JetValve Catheter-Based Deployment and Crimping.** Transcatheter delivery involved fixing the scaffold in a self-expanding nitinol stent for transapical placement via entry through the right ventricle (RV) and positioning via a guidewire system, deployment of the stented scaffold over the native leaflets located proximal to the pulmonary artery (PA), and retraction of the catheter through the ventricle as the radial pressure of the released stent held the valve scaffold in place (a). The preparation for this process involves anchoring hydrated scaffolds into nitinol stents and crimping from the 30 mm fully extended size to the fully crimped 9mm size for delivery (b).

**3.2.11 Statistical Analyses**

For scaffold fiber diameter, scaffold porosity, and scaffold stiffness measurements, One Way Analyses for Variance between the biohybrid compositional groups were conducted using SigmaPlot (v12.0, Systat Software Inc.). For fiber diameter, Shapiro-Wilk normality failed, thus a Kruskal-Willis One Way Analysis of Variance Ranks Test was conducted; for pairwise multiple comparison among scaffold groups, a Tukey Test was conducted (diameter: \( n=6 \) samples per biohybrid condition, 10 FOV per scaffold). Scaffold porosity passed Shapiro-Wilk normality (\( P=0.954 \)) and equal variance (\( P=0.680 \)); for
pairwise multiple comparison among scaffold groups, a Tukey Test was conducted (porosity: n=6 samples per biohybrid condition, 6 FOV per sample). Large strain perpendicular and small strain parallel and perpendicular stiffness measurement failed Shapiro-Wilk normality, thus a Kruskal-Willis One Way Analysis of Variance Ranks Test was conducted; for pairwise multiple comparison among scaffold groups, a Dunn’s Method test was conducted (large strain perpendicular and small strain parallel and perpendicular stiffness: n=6 per biohybrid condition n=5 for native valve). For all statistical analyses, p-values less than 0.05 were considered statistically significant and all values reported as mean ± standard error of the mean.

3.3 Results

3.3.1 JetValve Biohybrid Structure and Mechanics

The JetValve micro-structure was designed to recapitulate the native valve ECM as a platform for endogenous remodeling. Fiber diameter and porosity [269, 270] are structural scaffold parameters that contribute to the degree of endogenous cellular attachment and infiltration during the remodeling process. To control these parameters, scaffolds were composed of biohybrid blends of poly-4-hydroxybutyrate (P4HB) and gelatin, i.e. denatured collagen, which is the primary structural component of valve leaflets. By varying the biohybrid concentration within the scaffold, fiber diameter [49] and alignment could be controlled to approximate that of the native valve ECM. We mixed high molecular weight P4HB (MW ~450 kDa) with gelatin (MW ~50-100 kDa) to control solution viscosity and consequently fiber diameter [48, 218]. By decreasing P4HB content while maintaining a constant extrusion speed of 30k RPM, fiber diameter could be reduced (range: 100/0 1.28 ± 0.39 μm to 20/80 68 ± 0.19 nm) to achieve a higher porosity (range:
100/0 41.59 ± 1.58% to 20/80 55.51 ± 2.38%) within the construct (Figure 3.4a). As in the native valve structural fibrosa, fibers were primarily oriented in the circumferential direction of the scaffold leaflets to withstand transvalvular loading during diastole while the high Mw of the P4HB and non-woven mesh structure would allow for elastic, radial stretching during systole. The continuous deposition of fibers onto mandrels angled 45° relative to the fiber extrusion plane produced circumferential alignment within leaflets, recapitulating the load bearing, collagen-rich fibrosa layer of the native valve ECM [271] (native OOP: 0.49 ± 0.06, 60/40 JetValve OOP: 0.44 ± 0.03, Figure 3.4b). As a proof-of-concept of the scaffold microstructure’s guidance of tissue formation, valvular progenitor cells seeded onto scaffolds in vitro, formed circumferentially aligned tissues mimicking the typical phenotypic structure of valvular interstitial cell tissues [272] (Figure 3.4b).

In addition to architectural cues, valvular progenitor fate during tissue development is strongly influenced by its mechanical environment [273]. Valvular endothelial cells [217], interstitial cells, and mesenchymal stem cells (a potential valvular progenitor cell type) have shown morphological and phenotypic dependency on substrate stiffness and stretch [274, 275]. We therefore designed the stretch-dependent, biaxial stiffness of our scaffolds to recapitulate that of the native leaflet ECM. The P4HB/gelatin ratio of fibers governed the biaxial stiffness of the bulk scaffold at both low (0-10%) and high (10-20%) strains (Figure 3.4c). At low strain, in the primary (circumferential) axis of fiber alignment, scaffold stiffness ranged from 505.52 ± 61.72 kPa (40/60) to 5.12 ± 0.82 MPa (100/0) vs. 643.23 ± 215.76 kPa (native); in the perpendicular (radial) axis of fiber alignment, scaffold stiffness ranged from 211.91 ± 31.32 kPa (40/60) to 4.33 ± 0.16 MPa (100/0) vs. 501.41 ± 74.72 kPa (native). At high strain, in the primary (circumferential) axis of fiber alignment,
scaffold stiffness ranged from 1.16 ± 0.11 MPa (40/60) to 34.47 ± 1.54 MPa (100/0) vs. 3.33 ± 0.45 MPa (native); in the perpendicular (radial) axis of fiber alignment scaffold stiffness ranged from 338.38 ± 44.31 kPa (40/60) to 13.89 ± 0.75 MPa (100/0) vs. 1.49 ± 0.40 MPa (native). Only when the P4HB/gelatin ratio of fibers was reduced to 60/40 or 40/60 did scaffold stiffness approximate that of the native leaflet in both strain regimes in the primary and perpendicular axes of fiber alignment (Figure 3.4d). To encourage simultaneous endogenous remodeling and scaffold degradation once implanted, gelatin was not crosslinked. As a result, scaffold blends with less than 40% P4HB content lost mechanical integrity once hydrated in PBS at 37°C, mimicking the physiological environmental conditions that scaffolds would be subjected to once implanted.

3.3.2 JetValve Surface Biochemistry and Hydrated Shelf Life

Because JetValve biohybrid scaffolds were physically blended, the compositional effects of not crosslinking and hydrating of the scaffold were further analyzed due to the time required for minimally invasive surgical delivery preparation. To prepare for stent anchoring and crimping, JetValves are hydrated, resulting in more pliable fibers. Because the scaffold protein content was not crosslinked, once hydrated the shelf-life of the scaffold would be limited due to passive gelatin diffusion from the surface of the fibers. Using FTIR, initial relative protein content and homogeneity of RJS, and electrospun for comparison, biohybrid fibers was measured by comparing the height of the carbonyl stretch peak (1720 cm\(^{-1}\), indicative of P4HB) with those of the amide I and amide II peaks (1645 and 1535 cm\(^{-1}\) respectively, indicative of gelatin, Figure 3.5) [49].
Figure 3.4: Biohybrid Structural and Stiffness Characterization. At a 30k RPM fiber extrusion rate, 4% w/v P4HB/gelatin solutions had decreased fiber diameter but increased percent scaffold porosity as a function of increased protein content (a, scale bars 25 μm surface and 50 μm X-section, n=6 scaffolds per condition, *p<0.5). At a 45° 3k RPM mandrel collection rate, the fiber anisotropy of 60:40 scaffolds recapitulated the collagen alignment of native leaflets as measured by OOP (b, scale bars 50 μm, n=16 scaffolds, n=5 native leaflets); mesenchymal stem cells (MSC, a potential valvular progenitor cell type) seeded onto aligned scaffolds formed anisotropic tissue in the direction of fiber alignment (scale bars 50 μm for SEM and stain, blue nucleus and white f-actin). Increasing protein content of spun scaffolds decreased the low strain (0-10%) and high strain (10-20%) biaxial global stiffness of scaffolds (c, n=6 scaffolds per condition, *p<0.5). 60:40 and 40:60 P4HB/gelatin blends matched native leaflet stiffnesses in these biaxial (blue parallel, red perpendicular to fiber alignment) strain regions (d, scale bar 5 mm, n=6 scaffolds per condition, n=5 native leaflets; *p<0.5).
To investigate prepared JetValve shelf life, the 60/40 P4HB/gelatin scaffold blend was chosen as it best mimicked the leaflet structural and mechanical design criteria described above while maintaining constant polymer crystallinity and therefore a predictable degradation profile over a range of manufacturing spinning speeds (Figure 3.6a and Figure 3.6b). The as-spun, dry composition of the 60/40 JetValve blend was precisely

Figure 3.5: Surface Protein Content of RJS and Electrospun Fibers. FTIR spectra of RJS fibers (a) and electrospun fibers (b) after soaking in ultra-pure water over 7 days. As a proof of concept, 75/25 PCL/Gelatin fibers were used to determine the relative rate of protein loss of uncrosslinked biohybrids in water. The ratio of amide I/II:carbonyl peaks was compared to quantify the protein:polymer ratio in RJS and electrospun (ESP) fibers in time (c). Imaging FTIR of biohybrid fiber bundles at t = 0, 1, and 24 hr (d) was used to determine the homogeneity of fiber surface content during soaking. Red represents highest absorbance (thickest portion of the fiber bundle) while blue represents lowest absorbance (scale bars 30 mm)
measured to be 58.43 ± 2.30% P4HB, 39.85 ± 1.86% gelatin, and 0.44 ± 0.4% HFIP using. Once hydrated, the surface gelatin composition of the scaffold increased to 56.42 ± 0.81% but returned to 43.48 ± 0.89% after 7 days in pure water (XPS, Figure 3.6c). As fibers within the scaffold were hydrated, uncrosslinked and therefore chemically unbound gelatin first diffused to the fiber surface, then into the surrounding water. However, after 1 week, the bulk biaxial stiffness of the scaffold was measured to be only slightly increased (not significantly significant) despite the initial loss of diffused gelatin (Figure 3.7). Additionally, the HFIP content decreased by nearly half (0.26 ± 0.07%) after 1 day in water suggesting that the residual solvent was only present in trace amounts once hydrated, no toxic effect of hydrated scaffold were observed. The hydrated shelf life of the hydrated 60/40 JetValves was therefore approximatively 1 week, the timeframe within which JetValve composition stayed within 60/40 to 40/60 P4HB/gelatin composition.

3.3.3 aRJS Manufactured JetValve Batch Process Capability

In order to evaluate scaffold quality and fabrication reproducibility, essential for clinical translation [257], we implemented standard industrial manufacturing process controls based upon the JetValve design criteria described above. We accomplished this by analyzing the structural, mechanical, and biochemical batch process capability (C_p) of each scaffold as a measure of both manufacturing accuracy and precision [267]. Batch C_p specifications were held to standard precision values of ±3 times the batch parameter variance, ensuring that over 99% of scaffolds within a passing batch were within specification. Despite laboratory-scale batch sizes (≤4), manufacturing achieved higher than 70% batch acceptance rate, which was increased to 100% with the addition of small batch correction factors [276] (Figure 3.8).
Figure 3.6: Biohybrid Crystallinity and Prepared Shelf Life. Polyester crystallinity peak shift as a result of protein content and extrusion speed (a and b, n=4 scaffolds per condition One Way Analyses for Variance, Tukey Test; *p<0.5 between condition and 20/80 P4HB/gel, #p<0.5 between condition and 40/60 P4HB/gel, †p<0.5 between condition and 60/40 P4HB/gel, ‡p<0.5 between condition and 80/20 P4HB/gel). XPS ratiometric analyses of hydrated scaffolds mimicking prepared scaffolds revealed a gradual leaching of uncrosslinked gelatin from the surface of the scaffold over the course of 1 week as the gelatin diffused to the surface of the fiber and then into the water (c, n=3 scaffolds per condition). Only trace amounts of HFIP solvent remained detectable upon hydration suggesting a 1 week shelf life is available for prepared scaffolds without the need for specific solvent removal post processing.
Figure 3.7: Biaxial Stiffness at 1 Week Hydration. 60/40 P4HB/Gelatin JetValve scaffolds were hydrated in Ultra-Pure water for 1 week to simulate preparation for surgical implantation; scaffolds must be hydrated to suture into stents and crimp for transapical implantation. 1 week represents an exaggerated time for preparation as the JetValve spinning process is designed for ‘on-demand’ manufacturing and the suture/crimp preparation is typically done on the order of hours, not days unless there is a delay in operating time. 7 day soaked scaffold (X-7d and Y-7D above) were measured to have a slight increase in stiffness compared to “Fresh” or as spun scaffold (stiffness measurements were not significant in the primary, X, or perpendicular, Y, axes of fiber alignment; n=3 scaffolds per condition).
Figure 3.8: Batch Process Capability Quality Control. Batch process capability (a, CP) was calculated for each batch of JetValves made taken from sample strips incorporated into the scaffold manufacturing process (dashed lines). Acceptable manufacturing Upper Control Limits (UCL) and Lower Control Limits (LCL) were set for each capability criteria (see Materials and Methods); CP is the minimal ratio of the distance from the mean (µ) of a design criteria value to each control limit and the allowable variance (σ), taken as 3 standard deviations away from the mean in industrial manufacturing. A CP of 1 or greater is taken as acceptable (green shading) while a CP less than 1 (red shading) in any tested design criteria results batch reject due to high variance and (i.e. manufacturing inconsistency). The CP report of the JetValves made for implantation (b, n=7 scaffold batches, evaluation of scaffold structural, mechanical, and compositional design criteria parameters).
3.3.4  *In vitro and In vivo* JetValve Functional Testing

JetValve functionality was evaluated both *in vitro* and *in vivo*. Preliminary evaluation of scaffold function using an *in vitro* pulse duplicator system was conducted to ensure the integrity of the scaffold leaflets under physiologic pressures and flows. JetValves were continuously loaded for 48 hr under pulmonary conditions and maintained good leaflet motion and competence with less than 20% regurgitant fraction (Grade I) [277] during diastolic closing (Figure 3.9a). The *in vitro* durability and competency of JetValve scaffolds demonstrated acute structural and functional efficacy requisite for safe implantation studies.

*In vivo* studies were then conducted to determine if 1) JetValve scaffolds could be delivered using a minimally invasive method, 2) if the as-spun, acellular scaffolds would be immediately functional upon implantation, and 3) if the scaffold design was biocompatible (i.e. non-thrombogenic). JetValves were delivered transapically into the native pulmonary valve position in an ovine model for 15 hr as a proof-of-concept. Using angiography, loaded catheters were positioned for scaffold-stent deployment in the orthotopic pulmonary valve position. As with *in vitro* testing, implanted scaffolds revealed acute functionality as sufficient leaflet motion and good coaptation area were observed with non/minor regurgitation fraction on echocardiography. Transvalvular pressure gradients across the valve, <2 mmHg, were also comparable to native leaflets during systole (Figure 3.9b). Gross examination of histological analyses done at the time of explantation revealed competent valves with pliable and intact leaflets (Figure 3.9c). No/minor thrombus formation and initial cellular attachment on the scaffold surface were observed (Fig 3.8c) indicative of the acute safety and compatibility of JetValve scaffolds.
JetValve functional performance (pulmonary conditions) was evaluated prior to implantation studies using a pulse duplicator system (a): pressure and flow recordings at 48 hr, arterial view images during systole (opening, white) and diastole (closing, blue). Arterial pressure varied between ~15 and ~40 mmHg while ventricular pressure varied from ~15 and ~30 mmHg (FDA Waveform, Vivitro Inc.). Flow through the JetValve reached ~145 ml/s during peak systole with complete valve closure during diastole (<20% regurgitant fraction at the beginning of diastole prior to no flow at closure). Three-dimensional echocardiography from the pulmonary artery revealed complete leaflet opening and closing during systole and diastole of implanted JetValves. Doppler imaging confirmed unrestricted blood flow through the sufficiently open leaflets during systole and good coaptation area with minor regurgitation fraction during diastole (b). Explanted scaffolds at 15 hr revealed no/minor thrombus formation on the leaflets and initial infiltration of neutrophils were observed (c, scale bars: 10 mm pulmonary view, 20 mm cross sectional view, 2 mm cross sectional histology, and 100 μm histology inset).
3.4 Discussion

In this study, we manufactured JetValves with fibrosal-like fiber alignment and global semilunar valve structure in minutes. This level of throughput is an improvement in time to manufacture relative to previously reported mandrel-based, fibrous valve fabrication techniques [278, 279]. Slow solution infusion rates (≤2 ml/hr) and grounded-mandrel collection speeds (≤1 RPM) have resulted in production times ranging from 1.5 hr [278] to over 3.5 hr [279] per scaffold using comparable electrospinning methods. By increasing infusion and fiber collection rates by two and three orders of magnitude respectively, we were able to produce 30 mm diameter JetValves in less than fifteen minutes. JetValve production times can be compared to those achievable with 3D printing techniques [280, 281]; however, 3D printing still remains unable to produce the spatial resolution of nano/macro-fiber production platforms. Similarly, hydrogel and soft-polymer molding and patterning techniques for engineering heart valves have been limited to feature resolutions within tens to hundreds of microns [282-285] and often require hours to days for fabrication due to gelation/layer bonding times. In comparison, we were able to achieve fiber resolution ranging from hundreds of nanometers to microns by simply varying solution composition in order to rapidly recapitulate the size scale and alignment of the native fibrosa ECM layer of the valve.

Scaffold mechanics and biochemical properties are critical for guiding specific tissue repair and regeneration [286, 287]. As we and others have shown, individual fibers can be designed to exploit the cell’s ability to transduce mechanical forces through nanoscale adhesion to the ECM network [288-290] dependent on the fiber biochemistry [101, 291, 292]. Accordingly, bulk JetValve stiffness was engineered to match that of native tissue
similar to previously reported ‘off-the-shelf’ cell-based manufactured valve scaffolds [222, 252] but without the need for long culture and in vitro conditioning. JetValve stiffness values were an order of magnitude smaller than previously reported, as-spun off-the-shelf fibrous scaffolds [293] and commercially available ‘stiff’ bioprosthetic valves (single versus tens of megapascals) [294]. This was made possible by the inclusion of uncrosslinked gelatin in the biohybrid JetValve fibers. Despite not crosslinking JetValves, exposure of biohybrid fibers to water for up to 1 week did not significantly change the stiffness of the scaffold. During this period, P4HB/gelatin content remained within compositional range for mimicking the measured native tissue stiffness values. This length of hydrated shelf life is comparable to recent fiber/gel valvular scaffold composites [295] and is well in excess of the time required for pre-implantation procedures: on the order of hours for stent fixation, crimping, and surgical delivery preparation.

While recent studies have begun to apply defined standards (e.g. ISO) for the assessment of tissue engineered heart valve functional performance [295, 296], little work has been done to establish industrial style quality control standards for tissue engineering processes and products [297]. Therefore the structural, mechanical, and compositional scaffold design parameters discussed above were measured and evaluated for each JetValve prepared as a factor of safety for the animal models used in functional implantation testing. The automated method of JetValve assembly and simplicity of materials customization enabled straightforward implementation of multi-parameter process capability metrics [291, 298]. Our group has developed similar multi-parameter quality assessment indices for stem cell manufacturing [60, 103] and suggest that the same can and should be done for tissue engineered products [261]. We envision batch process
capability and other quality control metrics may likewise be applied to future JetValve designs and similar automated in situ tissue engineered scaffold processes to ensure patient or model safety prior to functional testing.

Restoration of valvular functionality upon implantation is the immediate goal of regenerative scaffolds. As-manufactured JetValves showed competency and biocompatibility both in vitro and in vivo after crimping and minimally invasive delivery. Scaffold stiffness plays a critical role in leaflet kinematics and the development of transvalvular pressure gradients during systole [197, 299]. As in previously proposed polymer/gelatin composite models for achieving native tissue-like stiffnesses [300], the biohybrid JetValve scaffold composition was tuned to mimic native ECM stiffness. As a result, transvalvular pressure gradients of <2 mmHg were observed in vivo which is equivalent to recently published human and ovine tissue engineered heart valve (<5 mmHg) [268, 296]. However, in vitro pulse duplicator testing did reveal a regurgitant fraction of just under 20% which is slightly above ISO standards of similarly sized heart valves [295]. These data did not appear, though, to fully predict JetValve functionality in vivo where rapid and complete leaflet coaptation and minimal closing jet were observed with echo/doppler imaging. JetValves were mounted in the pulse duplicator system in the absence of radial loading which is normally applied in vivo by the surrounding tissue (pulmonary artery and valve annulus). This resulted in full expansion of the stent and may explain the level of measured regurgitation in vitro.

In addition to restoring function, the long term goal of all regenerative, in situ tissue engineered products is to promote both full regeneration and adaptive growth of the target tissue. Such scaffolds should serve as resorbable platforms onto and into which the body
can “auto-engineer” its own replacement tissue that, ideally, perfectly matches healthy native tissue. This requires intricate engineering design of scaffold architectural, mechanical, and biochemical properties [293] to control time-dependent biological processes including cellular recruitment and inflammation [301]. We suggest that inclusion of anti-inflammatory agents and/or growth factors is possible using the JetValve manufacturing method without significant modification to the technique or time to production. Growth factors associated with development such as transforming growth factor-β1 (TGF-β1), bone morphogenic proteins (BMPs), and/or platelet-derived growth factors (PDGFs) may be incorporated to elicit endothelial-to-mesenchymal transformation (EMT), for example, in order to populate and remodel the scaffold [291, 302, 303]. Additionally, recently reported hybrid-manufacturing techniques for the production of more complex, tri-layered scaffolds have been developed in an effort to better mimic this stratified structure of the valvular ECM for optimal hemodynamic performance [295, 304-306]. The JetValve production platform is amenable to fabrication of similarly layered scaffolds while maintaining industrial-like scaffold production rates [307-309] due to its additive approach. By successively spinning fibrosa, spongiosa, and ventricularis-like layers, stratified JetValve production will be possible.

We further propose that this method can be valuable for the fabrication or other fibrous replacement organ scaffolds or for production of customized scaffolds given the specific age, size, and organ or organ-part needs of a patient [261]. Fibrous, vascularized tissue such as myocardium or branching blood vessels may incorporate vascular endothelial growth factors (VEGFs), angiopoietins, or ephrins to elicit neovascularization within the scaffold [310]. Although precise growth factor combinations and amounts
needed to recruit tissue specific cell progenitors remains to be identified [257, 311], the flexibility of the JetValve manufacturing process described here would allow for easy incorporation of multiple factors into scaffolds to promote homing and assembly of endogenous cells and tissues. Furthermore, because mandrels can be 3D printed in any shape, scaffolds of customized anatomy can be spun using this technique: combining the JetValve mandrel-based spinning process with the idea of 3D printing patient specific organ geometries [281, 312-314]. The manufacturing process we propose is well suited to rapidly and iteratively study the properties and compositions needed for fibrous scaffold-based endogenous tissue repair and is amenable to patient-specific customization.

3.5 Conclusion

In this study, we have introduced a manufacturing process for the rapid fabrication of fibrous, semilunar heart valve scaffolds. By varying manufacturing parameters such as solution composition, extrusion/collection speed, and mandrel size or spin angle, functional scaffolds were built to recapitulate the basic native valvular ECM with the goal of eliciting endogenous repair mechanisms once implanted. This method is amenable to further customization by tuning the same multiscale structural, mechanical, and biochemical scaffold parameters to match the anatomy of specific patients. Because scaffolds were fabricated three-dimensionally, they could easily be incorporated into stents and implanted minimally invasively as-spun, without the need for post processing or in vitro preconditioning. The simplicity and control of this scaffold manufacturing process offers a viable, cell-free and clinically translatable alternative for the fabrication of heart valves and other fibrous organs.
4 Conclusions

4.1 Overarching Themes and Strategy

The overarching theme of this research is that the fundamental processes that initiate tissue formation and degeneration determine the design criteria and constraints of materials-based regenerative therapies. In this dissertation we have taken a bilateral approach to investigating this idea with respect to valvular tissues by asking both basic tissue and translational material science questions. We have focused on determining the basic spatiotemporal scales at which fibrosis progresses in cardiac valves with particular focus on the acute structural and functional effects on the fibrosa layer of the leaflet. This work has led to the development of in vitro technologies designed to help clarify the role of contractile and secretory valvular cell types (interstitial cells) in early drug-induced pathology. Based upon lessons learned in vitro, we have begun to engineer platforms for building biomimetic valvular scaffolds to restore functional tissue in vivo. Because the valvular ECM is principally fibrous, our work has focused on designing biomaterials manufacturing processes with the ability to produce nanofiber-based, three dimensional scaffolds. Together with our collaborators, we are currently in preclinical testing for our fibrous heart valve prosthesis “JetValve”, presently developing the technology further, beyond the scope of this dissertation, and creating a commercialization strategy.

4.2 Engineered Microphysiological Tissues for Modeling Disease

Purpose: Microphysiological tissues, commonly referred to as “Organs on Chips,” are engineered tissues and microenvironments designed to study basic questions surrounding tissue formation and disease/drug progression and effect [241]. Building cardiac valve-based microphysiological tissue assays are of particular interest to our
research because there remains a lack in understanding of early tissue-level fibrotic behavior. Valvular fibrosis is generally considered to be a chronic condition, clinically presenting in late stage functional insufficiencies. As a result, early diagnosis is difficult: we lack the tools to study what clinicians should be looking for. To date, most researchers in the field have focused on standard cell culture techniques to study valve fibrosis \textit{in vitro}. Conventional studies include investigating cellular aggregation, mitogenic activity, or the degree of myofibroblast activation of diseased cells grown on tissue culture plastic or glass. However, these diseases are defined clinically by degeneration at the tissue-level: reduced tissue anisotropy and stiffening of the valvular ECM for example. Because we know that the mechanical and biochemical environment of cells and tissues determine their function, we were motivated to build upon current methods to study this disease progression on substrates engineered to recapitulate the native tissue using readouts that are clinically relevant.

\textit{Approach}: To study the development of acute valvular fibrosis \textit{in vitro}, we have developed two complementary microtissue assays to determine early changes in tissue structure and function. As a case study, we applied the drug Pergolide, a valvulopathogen known to cause severe valve fibrosis clinically, to engineered valvular interstitial cell (VIC) tissues \textit{in vitro}. We found that VIC tissues built on thin films lost axial alignment and the capacity to generate tissue stresses after just 24 hours of drug treatment. These tissues displayed phenotypic markers indicative of a more motile and synthetic cell type, rather than highly contractile as has been shown in histological analyses of chronically exposed valve tissue. On fibrous substrates that were mechanically and chemically tuned to mimic the native healthy valve ECM, drug treated VIC tissues became biaxially stiffer, notably in
the direction of fiber alignment [271]. With these experimental insights, we were able to identify an early role of VICs as modulators of tissue tone, structure, and mechanics during the onset of disease. Early drug-induced valvular fibrosis appears to be, at least in part, due to an overly synthetic activation of VICs, thus diminishing their ability to generate tissue-level stresses. In previous studies, we observed that VIC spreading behavior is also determined by matrix stiffness in combination with stretch [275]. Taken together, these data suggest that fibrotic-like behavior in VIC tissues occurs early in the disease progression and may be a result of an inability to generate a healthy level cellular tension and consequently tissue tone.

Moving Forward → Our in vitro data highlight that a capacity for VICs to generate a level of cellular and tissue tone is required for health, loss of this capacity appears to be an early indicator of pathological fibrosis. Accordingly, and in the scope of our work, this suggests biomaterials for valve regeneration should be engineered with VIC stress generation and matrix synthesis as major design considerations. Many scaffolds designed for cardiac valve tissue engineering have been observed to suffer severe valve leaflet compaction and thickening during chronic in vivo remodeling. Accordingly, we have begun to design our fibrous scaffold leaflets with deeper cusps and slightly increased thickness to account for VIC remodeling during neotissue formation. Moving forward, we plan to broaden our focus to include valvular endothelial cell (VEC) tissue formation and remodeling. VECs form a continuous, anti-thrombogenic monolayer around the valve leaflets, providing a selective barrier that influences VIC behavior within the leaflet. In both valve formation and degeneration, VECs can undergo Endothelial-to-Mesenchymal Transformation (EMT) to populate and remodel the leaflet matrix. How do VECs form
healthy, functional monolayers? What mechanical and chemical cues drive EMT in health and disease? Can we control EMT to engineer fully cellularized replacement valves?

4.3 Manufacturing Biomaterials for Regenerative Tissue Engineering

Purpose: For patients requiring heart valve replacement, tissue engineering (TE) strategies have shown potential as permanent, regenerative solutions in comparison to current practices. Standard mechanical valve replacements require adherence to daily antithrombogenic medications while animal-sourced valves degrade over time; children requiring replacement valves will undergo numerous, invasive surgeries throughout their lifetime as neither option grows or adapts to the patient. The traditional tissue engineering paradigm is one founded in cell-based manufacturing. Appropriate cell populations, tissue-specific progenitor or stem cells for example, are grown on scaffolds and conditioned to form tissues in vitro that can then be implanted into the patient. This strategy has resulted in limited clinical application due to the complex logistics of production. Heart valve cell/scaffold conditioning times using this approach, for example, can take weeks or months. We and others are taking newer, more translatable approach to tissue engineering. By shifting focus to engineering biomimetic-scaffolds that can promote endogenous repair, we aim to reduce in vitro manufacturing time and cost in an effort to reach more patients [261].

Approach: To build biomimetic valve scaffolds, we have automated our ‘cotton candy-inspired’ Rotary Jet Spinning (RJS) fiber production platform to enable rapid and customizable heart valve fabrication. By developing a system for the controlled collection of fibers produced by our RJS technique, we were able to recapitulate semilunar valve structure from the micro- to the global tissue-scale. We have shown that the bulk biaxial
stiffness of our scaffolds can be controlled and tailored to match that of the native valve leaflet ECM, providing the strength required to withstand physiologic transvalvular pressures. Important for the eventual translation of this technology, we have shown that the structure, mechanical properties, and composition of the scaffolds are stable for up to 1 week once hydrated in preparation for implantation. In vitro flow testing under pulmonary conditions has led to ongoing in vivo functional testing performed in an adult sheep model. Minimal closing volume during diastole and low transvalvular pressures during systole where observed in vivo at acute time points. Initial explants at one month time points reveal endothelialization of the leaflet surface and early cellular penetration into the scaffold. We have further developed this system to build both fibrous ventricle models for drug testing and large blood vessels currently being tested in rodents. Taken together, we have demonstrated the versatility of our automated jet spinning and collecting technique for building cardiovascular tissue scaffolds.

Moving Forward → Our ability to quickly produce functional valves has resulted in new resources for the ongoing development of the scaffold material functionalization and construction. We are now studying the specific manufacturing parameters and fiber compositions that will enable adequate scaffold porosity and chemistry for cellular integration while maintaining overall valve functionality upon implantation. Furthermore we are developing more complex aortic valve scaffolds shapes (with sinuses) as well scaling production of our ventricle and vessel models. From this work, we have become interested in integrating the capabilities of 3D printing with fibrous scaffold manufacturing, combining the geometric versatility of printing systems with the nano-resolution of fiber production. We plan to build on the idea of 3D printing patient-specific organs to include
fibrous, cardiovascular constructs.

4.4 Future Research Directions

Our future research plans include continuing this bilateral approach to building tissues as outlined in this thesis: using fundamental lessons learned from basic science to inform how we engineer replacement tissues. Although much of the work presented here has focused on how to build heart valve tissues, our goal moving forward is to both go into more depth in this area while beginning to broaden the approach to other cardiovascular tissues.

1. Microphysiological Systems: Although the majority of microphysiological tissue “chips” have been engineered as two dimensional models, our physiology is three dimensional. Studying the interaction of multiple cell types in 3D environments during health and disease will give us more insight into how to build tissue replacements. We need to build organ chips bigger and better to obtain more relevant data from them. In the case of the heart valves, we plan to investigate the three dimensional mechanics (stiffness, flow, shear, etc.) and chemistry (growth factors, ECM composition, scaffold degradation rates, etc.) of how endothelial layers form, stabilize and functional in health, and degenerate in disease. This research will inform how we build more complex scaffolds that initiate endothelialization in order to reduce thrombosis and immune reaction while simultaneously, possibly, potentiating a healthy remodeling process. By applying similar principles to engineering vascular tissues, for example, we can study how the delivery of nutrients, toxins, or drugs to many other tissues is dependent on the health of the exposure route.
2. Biomaterials Manufacturing: Innovative biomaterials manufacturing approaches are needed for tissue engineering to truly impact the clinic. Much of the genetic cell editing and customization methods that capture most of the headlines today (CRISPR, iPS cells, etc.) will require matrices and delivery mechanisms of equal complexity and performance. How do we do this? Could we build materials that do not require these complex genetic editing or cell strategies? Our hypothesis it that that nature is the best builder – to build tissue matrices artificially, we should study and attempt to mimic how it is done in nature. Many of our tissues, including cardiovascular tissues, are composed of layer-specific fiber or gel-like matrices of unique structure and composition. Our plans are to merge rapid fiber production with 3D printing techniques to produces this level of complexity in scaffolds. In the case of heart valves, developing systems for directed fiber and gel deposition will enable the production of matrices that mimic the tri-layer structure of the native leaflet. Will these materials and matrices better function in vivo compared to our current, simpler models? Will they better initiate cell infiltration and regeneration? How do we evaluate this? For this work, incorporating chemists and mathematicians will be crucial. We need to understand how the proteins and polymer building materials that we use will unfold, assemble, crystalize, and degrade in the body. Similarly, modeling the parameter space of the fabrication processes from first principles is how we will gain control over manufacturing with the goal of customizing patient-specific matrices in the future. These are questions are the materials and manufacturing challenges that continue to surround the field of tissue engineering; we aim to begin addressing them in our future
scaffold work.

4.5 Future Challenges for Engineering Cardiovascular Tissues

Model tissue components that recapitulate portions of cardiovascular tissue structure and function have been engineered for both in vitro and in vivo applications. The design and build of these tissues are guided by what we observe and measure in the native tissue which likewise provides functional quality standards for their evaluation. The challenge moving forward is to establish goal-directed production based on clearly defined design criteria and benchmarks for success. To accomplish this, we must further define and determine scaling of the minimal, essential structures and functions of the native tissue that need to be recapitulated. Further, it is necessary to develop quantitative metrics that allow robust measurement and comparison of the parameters that define native tissue versus regenerative scaffold function. Computational algorithms allow quantitative assessment of traditionally qualitative measurements of biological form and function. Machine learning and statistical approaches for integrating the values from a variety of biochemical, structural, and functional experimental measurements into a single quality assessment score will allow comprehensive and reliable determination of engineered tissue quality [60, 102, 169]. As our knowledge of cardiovascular physiology grows, it would be of great utility to tabulate these data into a unified, digital repository. This could be used to take a systems biology approach to defining comprehensive, clinically-relevant criteria for guiding the design of engineered cardiovascular tissues generally.

Despite advances in our knowledge of the structure and mechanics of cardiovascular tissues, and improvements in the materials and production methods used to manufacture synthetic scaffolds, post-implantation monitoring of scaffold remodeling and performance
remains challenging. Assessment of cell infiltration, scaffold degradation, degree of vascularization and remodeling can be accomplished with histological analysis following explantation. However, this type of analysis often requires termination of the growing scaffold/tissue complex. Real-time, non-destructive imaging and functional analysis methodologies capable of assessing engineered tissue performance \textit{in situ} are needed to reveal the true time-dependent nature of the remodeling. Clinical diagnostic tools such as magnetic resonance imaging techniques (MRI) [225], computerized tomography (CT) [226], fluorescence imaging techniques [227], and echo/Doppler techniques may be useful to track scaffold/tissue growth and integration. Furthermore, fibrous scaffolds manufactured with dopants like magnetic or conductive nanoparticles can potentially be instrumented within the scaffold fibers during fabrication allowing for external, non-destructive monitoring of scaffold/tissue performance.

The need for vascularization strategies in tissue engineering was recognized early on [315] and remains a key challenge in the field [316-318]. Though not necessary in valvular tissues specifically, the initiation of healthy angiogenesis has been and continues to be a primary concern for any implanted cardiovascular scaffold, from amorphous hydrogels [319] to highly aligned fibrous scaffolds. If, during the remodeling of the scaffold, angiogenesis is not initiated, the thickness of the tissue formed will be restricted to the diffusion limit of oxygen and nutrients from the surrounding vasculature (~200 μm thick) [317]. To overcome this, scaffolds such as those designed for cardiac or skeletal muscle that require substantial thickness will benefit from the inclusion of angiogenic growth factors or initiators such as vascular endothelial growth factor (VEGF) to promote endogenous vascularization [320-322]. However, thicker scaffolds will not only need to be
chemically and biologically designed to initiate angiogenesis, but must also be physically designed to allow for vessel infiltration or sprouting. The porosity of a fibrous scaffold is therefore a critical design parameter for supporting vascularization of engineered tissues. For thicker scaffolds designed for myocardial repair for example, high porosity will be crucial for long-term, full regeneration. However, for tissues such as cardiac valves that must remain thin to be optimally functional, a balance between porosity levels high enough for cell infiltration but low enough to prevent vascularization will need to be achieved.

A similar balance and tuning of scaffold biochemical and mechanical properties will likewise need to be achieved to initiate an ideal inflammatory host response that will eventually promote healthy tissue regeneration. Defining the “healthy” level of inflammation is non-trivial: what is the minimal response to initiate endogenous tissue regeneration within the scaffold but not scarring or complete rejection? During the inflammatory phase of healing, matrix metalloproteinase (MMP) activation produces ECM fragments with potent pro-inflammatory actions affecting fibroblasts, endothelial cells, and leukocytes [323]. A key aim for cardiac regeneration strategies, for example, is the identification of signaling pathways that can be used to manage post-infarction inflammation, prevent excessive matrix degradation and attenuate adverse remodeling that leads to scar formation [324]. To accomplish this, anti-inflammatory, biologically-derived polymers [325] and synthetic anti-inflammatory drugs [326] have been incorporated directly into fibrous scaffolds. However, increasing the complexity of fibrous scaffolds by including more components, dopants, and cargo, may increase the risk for chronic inflammation and rejection. For example, instrumenting dopants such as conductive nanoparticles or nanotubes may cause chronic inflammation or toxicity [327], likely
dependent on where the scaffold is implanted. Controlling the inflammatory and angiogenic responses of the body are important design considerations of fibrous scaffolds moving forward and will require substantial investigation into the basic science of regenerative cellular processes. The advantage of using synthetically produced fibrous scaffolds compared to decellularized tissues is particularly evident here, as they are built from the bottom-up, adding only the components that are essential to a given application and can be thoroughly tested for their capacity to induce an immunological response.

In addition to these more ‘basic science’ design challenges, the industrial manufacturability of fibrous tissue engineered products is an important, often overlooked consideration that continues to limit their translation to the clinic. Manufacturing scalability, storage, and shelf-life optimization, as well as the establishment and implementation of quality control standards are hurdles for clinical translation and the development of reproducible *in vitro* assays. The importance of quality control and process validation, utilizing the scientific method to improve manufacturing process performance, was first proposed in the 1930s [328]. The implementation of statistics-based quality management practices over the decades since has led to substantial improvements in manufacturing efficiency and product quality in a wide range of manufacturing sectors because it provides insight about when a process is drifting out of control, a critical advantage over subjective, judgement-based assessment [329]. Statistical quality control inspection practices have been refined into a quality control paradigm commonly known as “Six Sigma” that has been successfully utilized to minimize the occurrence of product defects by holding process variation to one-sixth the difference between process mean and the upper/lower design specification limits [329]. Multi-parameter statistical quality
assessment strategies for stem cell manufacturing [60, 103] have already been implemented as well. We suggest the same can be done for fibrous tissue engineered products. Quantitative determination of standardized values for parameters such as fiber diameter, thickness, pore size, stiffness, and permeability will facilitate manufacturing and allow for standardized comparison. The adoption of these practices and standards will also accelerate products through the regulatory pathway if they are implemented early on in the development process. The translation of fibrous scaffolds into products with real patient impact will require a fusion of basic science and industrial manufacturing principles incorporated into the engineering design process.
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Chapter 1.1
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Chapter 1.2:
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Chapter 3

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6 List of Publications and Patents

6.1 Appendix A: List of Publications


[2] MacQueen LA, Sheehy SP, Chantre CO, Capulli AK, Park SJ, Goss JA, Campbell PH, Kossar FT, Parker KK. Functional tissue-engineered ventricle chambers evaluated by pressure-volume catheterization. [In Revision, PNAS]


### 6.2 Appendix B: List of Patents
